Inborn Errors of Metabolism with a Focus on Functional Analysis of a Special Mutation in *MCCB* Causing 3-Methylcrotonyl-CoA Carboxylase Deficiency, and *MMADHC* in Intracellular Vitamin B_{12} Metabolism, a Gene in which Mutations Can Lead to Three Different Phenotypes

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1 **Cryptic exon activation by disruption of exon splice enhancer: Novel mechanism causing 3-Methylcrotonyl-CoA carboxylase deficiency.**
Stucki M, Suormala T, Fowler B, Valle D, Baumgartner MR.
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2 **Gene identification for the cblD defect of vitamin B_{12} metabolism.**
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3 **Translational Initiation at Multiple AUGs can explain the three Phenotypes found in the cblD Defect of Intracellular Cobalamin Metabolism.**
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4 **A novel mutation in LMBRD1 causes the cblF defect of vitamin B12 metabolism in a Turkish patient.**
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J Inherit Metab Dis. 2010 Feb; 33(1):17-24.

5 **Spectrum of mutations in MMACHC, allelic expression, and evidence for genotype-phenotype correlations.**
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6 **Identification of a putative lysosomal cobalamin exporter altered in the cblF defect of vitamin B_{12} metabolism.**
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Personal contribution to the published works

1 cultivation of primary and transformed fibroblasts, transformation of primary fibroblasts, transfection of fibroblasts using retroviruses, lentiviruses, various chemical transfection reagents and electroporation, protein extraction, immunoblotting of protein extracts, optimization strategy for immunoblotting, carboxylase assays

2 design, cloning and preparation of all DNA constructs and vectors

3 cloning of all contstruct using patient mRNA or site-directed mutagenesis and linker PCR of existing constructs, cloning strategy, idea of mitochondrial targeting, idea of expressing truncated proteins, cultivation of transformed and primary fibroblasts, electroporation of fibroblasts, mRNA extraction, real-time PCR, sequencing, mutation analysis

4 cloning of the wildtype construct, linker PCR, subcloning

5 RT-PCR and cloning of patient mRNA to determine the allelic distribution of the three mutations found in *MMACHC* in each of these patients, sequencing, and mutation analysis

6 cloning of the wildtype construct, linker PCR, subcloning

PUBLICATIONS I

PERSONAL CONTRIBUTION TO THE PUBLISHED WORKS II

TABLE OF CONTENTS III

ABBREVIATIONS V

SUMMARY VII

ZUSAMMENFASSUNG VIII

INTRODUCTION

1 Inborn Errors of Metabolism 1

1.1 Amino acid disorders 1
1.2 Organic acidemias 2
1.3 Urea cycle disorders 2
1.4 Carbohydrate disorders 2
1.5 Mitochondrial disorders 3
1.6 Mitochondrial fatty acid disorders 3
1.7 Peroxisomal disorders 3
1.8 Lysosomal storage disorders 3
1.9 Others 4
1.10 Pathogenesis of inborn errors of metabolism 4
1.11 Manifestations and presentations 4
1.12 Symptoms that can be attributed to inborn errors of metabolism 4
1.13 Diagnosis of inborn errors of metabolism 5
1.14 Prenatal diagnosis of inborn errors of metabolism 5
1.15 Therapy of inborn errors of metabolism 5
1.16 Future aspects of the study of inborn errors of metabolism 6
1.17 Newborn Screening 6

2 Organic Acidemias 7

2.1 Metabolism of Branched-chain Amino Acids 8
2.1.1 Maple syrup urine disease (MSUD) 9
2.1.2 Isovaleric acidemia (IVA) 10
2.1.3 3-Methylcrotonylglycinuria (MCC) 11
2.1.4 Propionic acidemia (PA) 11
2.1.5 Methylmalonic aciduria (MMA) 12
2.1.5.1 Clinical findings in methylmalonic aciduria 14
2.1.5.2 Diagnosis of methylmalonic aciduria 15
2.1.5.3 Pathogenesis of methylmalonic aciduria 16
2.1.5.4 Treatment of methylmalonic aciduria 16
2.2 Vitamins with a focus on biotin and cobalamin 17
2.2.1 Introduction 17
2.2.2 Biotin (vitamin B_7) 18

2.2.2.1 Biotin deficiency 19
2.2.3 Biotin-dependent carboxylases and intracellular biotin metabolism 19
2.2.3.1 Acetyl-CoA carboxylase 21
2.2.3.2 Pyruvate carboxylase 22
2.2.3.3 Propionyl-CoA carboxylase 22
2.2.3.4 3-Methylcrotonyl-CoA carboxylase 22
2.2.3.4.1 3-Methylcrotonyl-CoA carboxylase deficiency 23
2.2.3.4.2 Newborn Screening and 3-methylcrotonyl-CoA carboxylase deficiency 25
2.2.3.4.3 Aims of the 3-methylcrotonyl-CoA carboxylase project 26
2.2.4 Cobalamin (vitamin B_{12}) 27
2.2.4.1 Cobalamin uptake and metabolism 28
2.2.4.2 Inherited and acquired cobalamin deficiencies 31
2.2.4.3 Methylmalonic acid and methylmalonic aciduria 33
2.2.4.4 Homocysteine and hyperhomocysteinemia 33
2.2.4.5 Aims of the cobalamin project 38

CHAPTER 1 **39**
Cryptic exon activation by disruption of exon splice enhancer: Novel mechanism causing 3-Methylcrotonyl-CoA carboxylase deficiency

CHAPTER 2 **52**
Gene identification for the cblD defect of vitamin B_{12} metabolism

CHAPTER 3 **75**
Translational Initiation at Multiple AUGs can explain the three Phenotypes found in the cblD Defect of Intracellular Cobalamin Metabolism

OUTLOOK AND CONCLUDING REMARKS **94**

ACKNOWLEDGEMENTS **96**

REFERENCES **97**

CURRICULUM VITAE **107**

ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AD	Alzheimer's disease
AdoCbl	5'-deoxyadenosylcobalamin (= adenosylcobalamin)
ALDH2	Aldehyde dehydrogenase 2
BCAA	branched-chain amino acid
C5OH	carnitine with a C_5OH substitution
Cbl	cobalamin
CBS	Cystathionine β-synthase
CNS	central nervous system
CoA	coenzyme A
CPS1	Carbamyl phosphate synthetase 1
ESE	exon splicing enhancer
EST	expressed sequence tag
ETF	Electron transfer flavoprotein
GC/MS	gas-chromatography / mass-spectrometry
HCS	Holocarboxylase synthetase
HPLC/MS	high pressure liquid-chromatography / mass-spectrometry
IEM	inborn error of metabolism
IVA	isovaleric acid / isovaleric aciduria
Lac	lactate
MCC	3-Methylcrotonyl-CoA carboxylase
MCEE	Methylmalonyl-CoA epimerase
MeCbl	methylcobalamin
MIM	mendelian inheritance in man (www.ncbi.nlm.nih.gov/omim)
miRNA	micro RNA
MLS	mitochondrial leader sequence
MMA	methylmalonic acid / methylmalonic aciduria
MS	Methionine synthase
MS/MS	mass-spectrometry / mass-spectrometry (= tandem mass-spectrometry)
MSR	Methionine synthase reductase
MSUD	maple syrup urine disease
MTHFR	Methyltetrahydrofolate reductase
MUT	Methylmalonyl-CoA mutase
NBS	new born screening
NIPD	non-invasive prenatal diagnosis
NMD	nonsense-mediated mRNA decay
OAA	oxalo acetate
ORF	open reading frame
PA	propionic acid / propionic aciduria
PC	Pyruvate carboxylase
PCC	Propionyl-CoA carboxylase
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PKU	phenylketonuria
Pyr	pyruvate

RDA	recommended daily allowance
ROS	reactive oxygen species
RT-PCR	reverse-transcription polymerase chain reaction
SAH	s-adenosylhomocysteine
SAM	s-adenosyl methionine
SDS	sodiumdodecyl sulphate
SUCL	Succinate-CoA ligase
TC I, II	Transcobalamin I, II
TCA cycle	tricarboxylic acid cycle (= citric acid cycle, = Krebs cycle)
TCblR	Transcobalamin receptor
THF	tetrahydrofolate
TMS	tandem mass spectrometry (= MS/MS)
UTR	untranslated region

SUMMARY

The present work focuses on two autosomal recessive inborn errors of metabolism, 3-methylcrotonyl-CoA carboxylase (MCC) deficiency, a defect in the catabolism of leucine, and on the cblD defect of intracellular vitamin B_{12} (cobalamin) metabolism.

MCC is a heteromeric mitochondrial enzyme composed of biotin-containing α (MCCA) and smaller β (MCCB) subunits encoded by *MCCA* and *MCCB*, respectively. We report studies of the c.1054G→A mutation in exon 11 of *MCCB* detected in the homozygous state in a patient with MCC deficiency. Sequence analysis of *MCCB* cDNA revealed two overlapping transcripts, one containing the normal 73 bp of exon 11 including the missense mutation c.1054G→A (p.G352R), the other with exon 11 replaced by a 64 bp sequence from intron 10 (cryptic exon) that maintains the reading frame and is flanked by acceptable splice consensus sites. In expression studies, we show that both transcripts lack detectable MCC activity. Western blot analysis showed slightly reduced levels of MCCB using the transcript containing the missense mutation, whereas no MCCB was detected with the transcript containing the cryptic exon. Analysis of the region harboring the mutation revealed that the c.1054G→A mutation is located in an exon splice enhancer sequence. Using *MCCB* minigene constructs to transfect MCCB-deficient fibroblasts, we demonstrate that the reduction in utilization of exon 11 associated with the c.1054G→A mutation is due to alteration of this exon splice enhancer. Further, we show that optimization of the weak splice donor site of exon 11 corrects the splicing defect. To our knowledge, this is the first demonstration of a point mutation disrupting an exon splice enhancer that causes exon skipping along with utilization of a cryptic exon.

In man, cobalamin is an essential cofactor in only two metabolic pathways. Intracellular conversion of cobalamin to its two coenzymes, adenosylcobalamin (AdoCbl) in mitochondria and methylcobalamin (MeCbl) in the cytoplasm, is necessary for the homeostasis of methylmalonic acid and homocysteine. We have identified the *MMADHC* gene responsible for the cblD defect that can cause isolated methylmalonic aciduria, isolated homocystinuria, or both. Various mutations are associated with each of the three biochemical phenotypes of the disorder. Using expression vectors containing the wildtype *MMADHC* with an enhanced mitochondrial leader sequence, mutations changing possible downstream sites of reinitiation or mutations introducing stop codons, we provide evidence that reinitiation of translation at downstream AUGs explains most of the phenotypes found in cblD patients. Furthermore, our data indicate that the sequence after Met^{116} is sufficient for MeCbl synthesis whereas the sequence between Met^{62} and Met^{116} is required for AdoCbl synthesis. Our findings support the hypothesis that a delicate balance exists between cytosolic MeCbl and mitochondrial AdoCbl synthesis, supporting the role of the MMADHC protein as a branching point in intracellular cobalamin trafficking.

ZUSAMMENFASSUNG

Die vorliegende Arbeit beschäftigt sich mit zwei autosomal rezessiv vererbten Stoffwechselkrankheiten, dem 3-Methylcrotonyl-CoA Carboxylase (MCC) Mangel, welches ein Defekt im Leuzin-Abbau ist, und dem cblD Defekt, einer Störung des intrazellulären Vitamin B_{12} (Cobalamin) Stoffwechsels.

MCC ist ein heteromeres mitochondriales Enzym aus Biotin-bindenden α (MCCA) und kleineren β (MCCB) Untereinheiten, welche durch *MCCA* und *MCCB* kodiert sind. Wir berichten über die Mutation c.1054G>A (p.G352R) in Exon 11 von *MCCB*, welche wir homozygot bei einem MCC-Patienten gefunden haben. Die Analyse der cDNA Sequenz von *MCCB* ergab zwei überlappende Transkripte: eines mit der Mutation c.1054G>A, sowie ein weiteres mit Ersatz der 73 bp von Exon 11 durch 64 bp aus Intron 10. Dieses kryptische Exon verändert das Leseraster nicht und weist akzeptable Spleiss-Stellen auf. Expressionsstudien zeigen, dass beide Transkripte keine MCC Aktivität besitzen bei nur reduzierter Proteinmenge beim mutationstragenden Transkript sowie Proteinverlust des Transkriptes mit kryptischem Exon. Die Mutation c.1054G>A ist Teil eines Exon-Spleiss-Enhancers. Transfektionsexperimente von *MCCB* Minigen Konstrukten in MCCB defizienten Fibroblasten zeigten bei Veränderung des Exon-Spleiss-Enhancers ein vermindertes Spleissen von Exon 11. Zudem konnte bei Sequenz-Optimierung der Exon 11-Spleiss-Stellen der Spleiss-Defekt korrigiert werden. Unseres Wissens ist dies der erste Nachweis eines durch Punktmutation in einem Exon-Spleiss-Enhancer resultierenden Exonverlustes bei gleichzeitiger Insertion eines kryptischen Exons.

Beim Menschen wird Cobalamin für nur zwei Reaktionsschritte des Stoffwechsels benötigt: als mitochondriales Adenosylcobalamin (AdoCbl) für den Abbau von Methylmalonsäure und als zytosolisches Methylcobalamin (MeCbl) für die Methylierung von Homocystein. Bei Vorliegen eines cblD-Defektes besteht eine Methylmalonazidurie, eine Hyperhomocysteinämie oder eine Kombination aus beiden Symptomen. Uns gelang die Klonierung des *MMADHC* Gens, welches für den cblD Defekt verantwortlich ist. Verschiedene Mutationen sind mit den drei Phänotypen des cblD-Defektes assoziiert. Durch Veränderung der mitochondrialen Importsequenz, durch Korrektur der zusätzlichen AUGs (Transkriptions-Start) und durch Einfügen weiterer Stop-Mutationen konnte gezeigt werden, dass eine Reinitiation der Transkription an weiteren AUGs die in cblD Patienten gefundenen Phänotypen erklären könnte. Weiter haben wir Hinweise darauf, dass ein cblD-Protein mit Beginn bei Met^{116} weiter MeCbl synthetisieren kann, und dass die Sequenz zwischen Met^{62} und Met^{116} notwendig für die Synthese von AdoCbl ist. Unsere Resultate bestätigen die Hypothese eines kritischen Gleichgewichts zwischen zytosolischer MeCbl und mitochondrialer AdoCbl Synthese. Das MMADHC Protein spielt somit eine wichtige Rolle bei der intrazellulären Cobalamin Verteilung.

1 Inborn Errors of Metabolism

The term “inborn error of metabolism” (IEM) was first used in 1908 by a British physician, Sir Archibald Garrod (1857 - 1936) while describing the nature and mode of inheritance of alkaptonuria, a disease in the metabolism of phenylalanine and tyrosine (1).

To date more than 500 IEMs are known (2). They occur relatively frequently (1:1'000 to 1:3'000 affected infants (3)) and, as a disease entity, are of considerable public health significance (2, 4). If undiagnosed and untreated, they can cause, depending on the corresponding disease, irreversible mental retardation, physical disability, neurological damage and fatality. For many IEMs, the lack of correlation between phenotype and genotype is one of many unresolved problems.

Consider a reaction converting substrate A to product B (1): In most IEMs, the problems arise due to 1) accumulation of the substrate (2), 2) the reduced availability of the product (3), 3) the production of another toxic metabolite C (4), or 4) a combination of 2-4 (Figure 1).

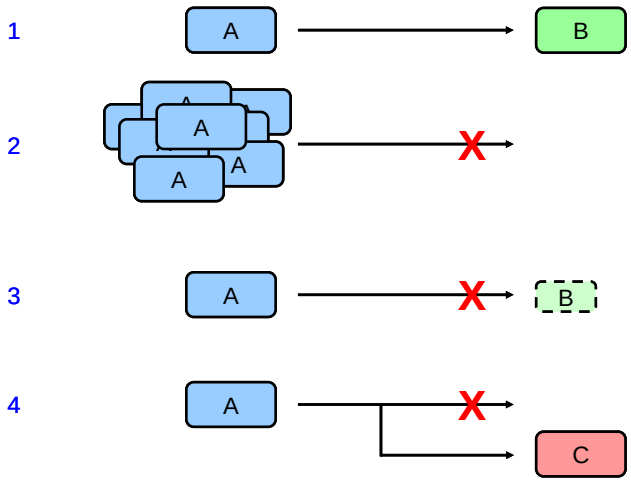


Figure 1: A schematic view of the most common problems arising from inborn errors of metabolism. If the conversion of A to B (1) is defective, problems can arise either due to the accumulation of A (2), the lack of B (3) or the production of another toxic metabolite C (4) or a combination of 2-4.

IEMs can be classified according to the type of metabolism involved:

1.1 Amino acid disorders

Amino acid disorders (amino acidopathies) are caused by defective catabolic pathways of amino acids. If there is an abnormal accumulation of amino acids in the plasma or an abnormal excretion of amino acids in the urine, the disorder is called either an “amino acidemia” or an “amino aciduria” depending on the major metabolite found. The symptoms of amino acid disorders typically result from an accumulation of the substance that cannot be metabolized. Often amino acid disorders present in initially well newborns that become acutely symptomatic after catabolic stress due to fasting or intercurrent infection or after a

change to a more protein rich feeding. Symptoms can include metabolic decompensation with lethargy and poor appetite and may progress to encephalopathy, coma and death, provided they are not recognized and treated promptly. Examples are maple syrup urine disease (MSUD, Mendelian Inheritance in Man (MIM) 248600) or tyrosinemia type 1. Other amino acidemias such as phenylketonuria do not present with acute symptoms but may lead to chronic progressive brain damage if they are not detected in the newborn period and treated promptly.

1.2 Organic acidemias

Organic acidemias (organic acidurias) are defined by an accumulation of abnormal acyl-CoA derivatives, which are usually toxic, as well as increased excretion of organic acids in the urine (5). Affected children develop life-threatening episodes of metabolic decompensation, poor feeding, vomiting and lethargy after an initial period of well-being. Later in life affected individuals may present with developmental delay or regression.

Examples of organic acidemias include methylmalonic acidemia (MMA, MIM 251000 and others) and propionic acidemia (PA, MIM 606054). For more detailed information on organic acidurias see section 2.

1.3 Urea cycle disorders

Urea cycle disorders are defects in the metabolic pathways transforming waste nitrogen into non toxic urea which can be easily secreted in the urine. Similar to amino acidopathies and organic acidemias these disorders often present in initially well newborns that become hyperammonemic after catabolic stress due to fasting or intercurrent infection or after a change to more protein rich feeding. As ammonia levels increase, infants quickly develop vomiting, hypothermia, irritability, lethargy, seizures and cerebral edema. Without intervention, they become comatose and die.

Examples of urea cycle disorders include citrullinemia (MIM 215700) and ornithine transcarbamylase deficiency (MIM 311250).

1.4 Carbohydrate disorders

Carbohydrate disorders are caused by defects in any of the pathways involved in the metabolism of carbohydrates. Affected children may present with lethargy and seizures due to hypoglycemia during times of fasting or with acute hypoglycemia leading to confusion, disorientation, coma and death (6). In other cases, these disorders can also lead to acute liver dysfunction, to chronic liver damage or to cardiomyopathy.

Examples of carbohydrate disorders include galactosemia (MIM 230400) and glycogen storage diseases like for example van Gierke disease (glycogen storage disorder type 1, MIM 232200).

1.5 Mitochondrial disorders

One of the hallmarks of mitochondrial disorders is elevated lactic acid which is usually increased due to a defect in either the tricarboxylic acid (TCA) cycle or oxidative phosphorylation in the respiratory chain. As many mitochondrial disorders have remained undiagnosed, the frequency by which they are now recognized increases (7).

The disorders can affect solely muscle, muscle and brain or multiple organs. Examples are pyruvate dehydrogenase deficiency, cytochrome C oxidase deficiency (MIM 220110) or deficiency of any other complex of the respiratory chain, mitochondrial DNA depletion syndrome, and many more.

1.6 Mitochondrial fatty acid oxidation disorders

Mitochondrial fatty acid oxidation plays an important role in the production of energy, especially during times of fasting (8): organic and fatty acids as well as amino acids are metabolized to acetyl-CoA within mitochondria. A condensation reaction with oxaloacetate then forms citric acid which is further oxidized in the TCA cycle. During times of low carbohydrate intake or fasting, affected children present with hypoglycemia, encephalopathy and lethargy. Cardiomyopathy and cardiac arrhythmias may also be present. Other findings may include hyperammonemia, metabolic acidosis. Examples include medium chain acyl-CoA dehydrogenase deficiency (MIM 201450), carnitine palmitoyltransferase deficiencies (MIM 255120, 600650) and mitochondrial trifunctional protein deficiency (MIM 600890).

1.7 Peroxisomal disorders

Peroxisomal disorders are characterized by a defect in one of the many functions carried out in peroxisomes. These include beta-oxidation of very long-chain fatty acids, the oxidation of dicarboxylic acids and bile acids, and the degradation of hydrogen peroxide by catalase. Clinical symptoms of peroxisomal dysfunction include dysmorphic facial features, microcephaly, hypotonia and sometimes neurological dysfunction. An example is Zellweger syndrome (MIM 214100).

1.8 Lysosomal storage disorders

Lysosomal storage disorders include defects in lysosomal degradation pathways of various compounds like glycoproteins, mucopolysaccharides and sphingolipids. Deficiency of a lysosomal enzyme leads to accumulation of partially degraded substrates. Thus the

lysosomes increase in size and are incapable of exerting their cellular function. The location and the extent of the occurring storage greatly influences the clinical manifestations. Symptoms may include progressive hepatomegaly, splenomegaly, short stature, neurological regression and many more (9). Examples include mucopolysaccharidoses, e.g. Hurler syndrome (MIM 607014) or sphingolipidoses, e.g. Fabry disease (MIM 301500), Gaucher disease (MIM 231000) and Krabbe disease (MIM 245200).

1.9 Others

Other classes of inborn errors of metabolism include purine and pyrimidine disorders, congenital disorders of glycosylation, porphyrias, steroid metabolism disorders and metal metabolism disorders.

1.10 Pathogenesis of inborn errors of metabolism

The pathogenesis of an IEM can generally be attributed to the loss- or gain-of-function of mutant proteins (usually an enzyme or a transporter). The genetic basis of IEMs is extremely heterogeneous and can involve any type of genetic defect. The disease is generally associated with altered metabolite flux through the pathway in which the mutant protein is involved. Importantly, net flux represents the integration of genotype, transcription, translation and environment which can make even out of “simple” monogenetic disorders complex diseases (10).

1.11 Manifestations and presentations

The enormous number of IEMs causes a wide range of manifestations and therefore nearly every presenting case may have a congenital metabolic disease as a possible cause; this is especially true in childhood.

Many deleterious effects of IEMs can be prevented, and long-term damage can be minimized, as long as the defects are diagnosed at an early stage. Therefore it is important to keep them in mind, especially when treating newborns and infants.

1.12 Symptoms that can be attributed to inborn errors of metabolism

First signs of an IEM can be poor sucking, feeding refusal, vomiting, excessive weight loss and abdominal distension. In such cases it may be adequate to start looking for IEMs. The following findings also corroborate the suspicion of an IEM:

- the pregnancy and delivery were both normal (maternal metabolism is not affected)
- the patient was well for the first few days of life (symptom-free period)
- the patient becomes ill during a catabolic state due to fasting or infection (intoxication)
- after each feeding the symptoms worsen (accumulation of substrate that cannot be metabolized)

1.13 Diagnosis of inborn errors of metabolism

IEMs should always be considered in the differential diagnosis when treating a newborn presenting with one of the above mentioned symptoms. The following laboratory studies can provide clues for the diagnosis: complete blood count, urine analysis, blood gases, serum electrolytes, blood glucose and lactate, plasma ammonia, reducing substances in the urine, urinary ketones, quantitative plasma and urinary amino acids, urinary organic acids and acylcarnitines in dried blood spots (11). Specific tests include enzymatic assays for specific enzymes from leukocytes, a skin biopsy (fibroblasts) or a tissue biopsy or necropsy (mainly liver or muscle). DNA sequencing is often used for confirmation and genetic counseling.

1.14 Prenatal diagnosis of inborn errors of metabolism

Many of the diagnostic techniques used for diagnosing IEMs in patients can also be used for prenatal diagnosis of IEMs. Metabolites can be measured in amniotic fluid and DNA isolated from chorionic villi can be used for sequencing. Also, cultured chorionic villi or amniocytes can be used for enzymatic assays. Because the collection of chorionic villi entails a risk of miscarriage, novel methods for non-invasive prenatal diagnosis (NIPD) are now being tested. They focus on enriching free fetal DNA that circulates in the plasma of the pregnant woman (12, 13). NIPD is established for fetal Rhesus determination as well as for fetal sex determination in women at risk of a sex-linked genetic disorder (14).

1.15 Therapy of inborn errors of metabolism

Many patients suffering from an IEM need to follow a strict dietary regimen lacking the substance that cannot be catabolized. During episodes of metabolic decompensation high dose glucose, insulin and lipids are used to prevent catabolism and promote anabolism. One of the few universal therapies available for the management of metabolic defects is dietary supplementation with L-carnitine and/or glycine. This enhances the body's endogenous ability to remove harmful, accumulated metabolites caused by a block in a metabolic pathway (15-17). Endogenous detoxification can be divided into two phases. Phase 1 reactions include oxidation, reduction and hydroxylation reactions that usually detoxify xenobiotic substances. Phase 2 includes the conjugation of xenobiotic and endogenous toxins (e.g.: to carnitine or glycine) in order to facilitate their urinary excretion as a conjugated metabolite (18-22). For some IEMs, especially for lysosomal storage disorders, enzyme replacement therapies are available (23).

For more than 20 years, gene therapy has also been investigated as a treatment option for IEMs, especially for phenylketonuria (PKU) (24). Recent results in mice showed that muscle-directed gene therapy using adeno-associated virus 2 could be a viable alternative treatment for PKU (25). Also other promising therapeutic strategies for the treatment of IEMs have

emerged recently such as chaperone therapies to enhance the stability of mutated proteins, compounds that modulate ribosome specificity thereby enabling the read-through of nonsense mutations, or antisense morpholinos inhibiting miss-splicing that is caused by intronic mutations leading to the inclusion of a cryptic exon into the mature mRNA (26-28).

1.16 Future aspects of the study of inborn errors of metabolism

With more and more knowledge on IEM's available, it is increasingly recognized that they are not mainly Mendelian traits but represent good examples of complex gene-environment interactions and, more specifically, gene-nutrient interactions leading to complex diseases (10, 29, 30). Furthermore, metabolism and detoxification of endogenous substances in individuals with IEMs may play a major role in determining the phenotype and prognosis. IEMs are therefore potentially powerful models to learn to dissect the even more complex multifactorial diseases such as common cancers, epilepsy, diabetes, multiple sclerosis etc.

In complex diseases, it has been proposed that a network of metabolite fluxes might be subtly altered to cause a phenotype (30). It is possible that an individual's genomic variant affects metabolite flux in a subtle fashion, without evidence of clinical disease. The cumulative effects of multiple sequence variations on a network of metabolite fluxes might, however, be sufficient to surpass the clinical threshold for disease, either alone or in combination with environmental factors (30, 31).

Recent findings in transcriptional research have shown that the transcriptome is considerably larger and more divergently expressed than was previously thought. For example small physiologically functional RNAs, miRNAs, are now known to be nested within intronic regions of primary RNA transcripts (32, 33). It is commonly accepted that they play important roles in post-transcriptional gene-regulation and are sensitive to environmental and dietary stimuli (34, 35). Their role in IEMs however remains to be determined.

1.17 Newborn screening

The origins of newborn screening (NBS) for IEMs can be tracked back some 75 years. In 1934, Asbjorn Fölling, a Norwegian biochemist, was asked to examine two severely mentally handicapped children of a former student. He found that they excreted high amounts of phenylketones in their urine (36). This was due to the inability to convert phenylalanine to tyrosine, a condition nowadays known as PKU. 25 years after this discovery, a phenylalanine-restricted diet became commercially available. Once this diet was available, asymptomatic infants with PKU that were identified and diagnosed during the first few weeks of life could be administered this diet in order to prevent permanent brain damage. Later it was shown that high levels of phenylalanine lead to disturbances in brain energy

metabolism, the creation of high energy phosphates and subsequently to neurotoxicity (37, 38).

In 1963, Robert Guthrie developed an assay that allowed screening of newborns for PKU: a few drops of blood collected on a filter paper within the first 2-3 days of life were used for rapid analysis of elevated phenylalanine (39).

The advent of novel experimental techniques, such as a range of mass-spectrometry methods, are heralding a paradigm-shift that will have a great impact on the quality of life of people with metabolic disorders (11). The US, Australia and many European countries have implemented extended population newborn screening programs using tandem mass spectrometry (MS/MS). With this approach it is possible to detect approximately 30 IEMs simultaneously at a presymptomatic clinical stage using the same filter paper card blood spots (40, 41). NBS programs revealed that the incidence of metabolic diseases is much higher than traditionally thought; in Australia for example the prevalence of IEMs was found to be around 1:6'500 (PKU excluded)(42).

As far more cases are diagnosed by NBS than previously by clinical symptoms, it remains to e clarified what proportion of detected individuals will become clinically symptomatic (42).

More than 40 years ago the World Health Organization has defined criteria for including a disorder into a (newborn) screening program (43). Accordingly, a disorder screened for should meet the following criteria:

- it causes significant morbidity and/or mortality
- an effective treatment is available
- there is some time before the onset of symptoms so that intervention can be effective
- the screening method and the assay is clinically validated
- the screening is economically “reasonable”
- the natural history of the disease is understood
- the incidence in the population to be screened is known and significant

Many of the disorders that are screened for today do not fulfill all these criteria. One example is 3-MCC that is discussed in section 2.2.3.4.

2 Organic Acidemias

Organic acidemias (organic acidurias) are characterized by the urinary excretion of organic acids. They are primarily the result of enzyme deficiencies in the catabolic pathways of amino acids, carbohydrates or the beta oxidation of fatty acids (44). The relevant pathways are depicted in Figures 2 and 4.

Affected babies are often full-term born babies that are initially symptom-free and then suddenly undergo relentless deterioration without apparent cause and are not responsive to symptomatic therapy. The reason for this rapid deterioration is the catabolic state of the

newborn in the first few days of life leading to accumulation of acyl-CoA derivatives that cannot be further metabolized followed by progressive intoxication. First signs include poor feeding and drowsiness that can develop into an unexplained progressive coma. Changes in the central nervous system (CNS) can include cerebral oedema and arousing suspicion of CNS infection. Further neuro-vegetative signs that may appear later include respiratory distress, hiccups, apnoeas and hypothermia. Additional phenotypes can include axial hypotonia, limb hypotonia, tremors, myoclonic jerks and hepatomegaly.

Typically, affected children have severe acidosis, ketosis and hyperammonemia. These findings can be accompanied by hypoglycemia and electrolyte abnormalities. Blood analysis can detect neutropenia, thrombocytopenia or pancytopenia due to bone marrow suppression. As early diagnosis and emergency treatment can prevent CNS dysfunction, it is required that toxins are removed immediately after diagnosis.

Initial treatment includes intravenous re-hydration and correction of acidosis, hyperammonemia, hypoglycemia and electrolyte abnormalities. To prevent further catabolism and promote anabolism patients are treated with high glucose, lipids and insulin. In addition, L-carnitine is administered to promote detoxification and to prevent secondary carnitine deficiency. Hemodialysis or hemofiltration may also be necessary in cases with severe acidosis or hyperammonemia (45). In methylmalonic aciduria (MMA) and propionic acidemia (PA) the administration of carglumic acid may be considered. The accumulation of propionyl-CoA in these disorders can lead to reduced synthesis of N-acetyl-glutamate, the physiological activator of the enzyme carbamyl phosphate synthetase 1 (CPS1). CPS1 plays an important role in the detoxification of nitrogen and can be activated by carglumic acid (46).

In survivors of the difficult initial decompensation a strict diet low in natural protein and supplemented with precursor-free amino acid mixtures and vitamins is essential to limit the formation of organic acid byproducts and enable normal growth. Phases of acute metabolic decompensation due to catabolic stress following intercurrent illness usually require intravenous therapy due to vomiting and nausea. During the acute phase protein is withdrawn from the diet for 24 to 48 hours, later a low-protein diet is gradually reintroduced (47, 48).

Prevention and early treatment of such recurrent episodes of metabolic imbalance is crucial. Metabolic derangement is potentially life-threatening and can be the cause of neurological sequels at any age.

2.1 Metabolism of branched-chain amino acids

Disorders in the degradation pathway of the three branched-chain amino acids (BCAA) leucine, isoleucine and valine are collectively termed branched-chain organic acidurias. The five most common ones include maple syrup urine disease (MSUD), isovaleric acidemia

(IVA), 3-methylcrotonylglycinuria (MCC), methylmalonic aciduria (MMA), and propionic aciduria (PA) (49).

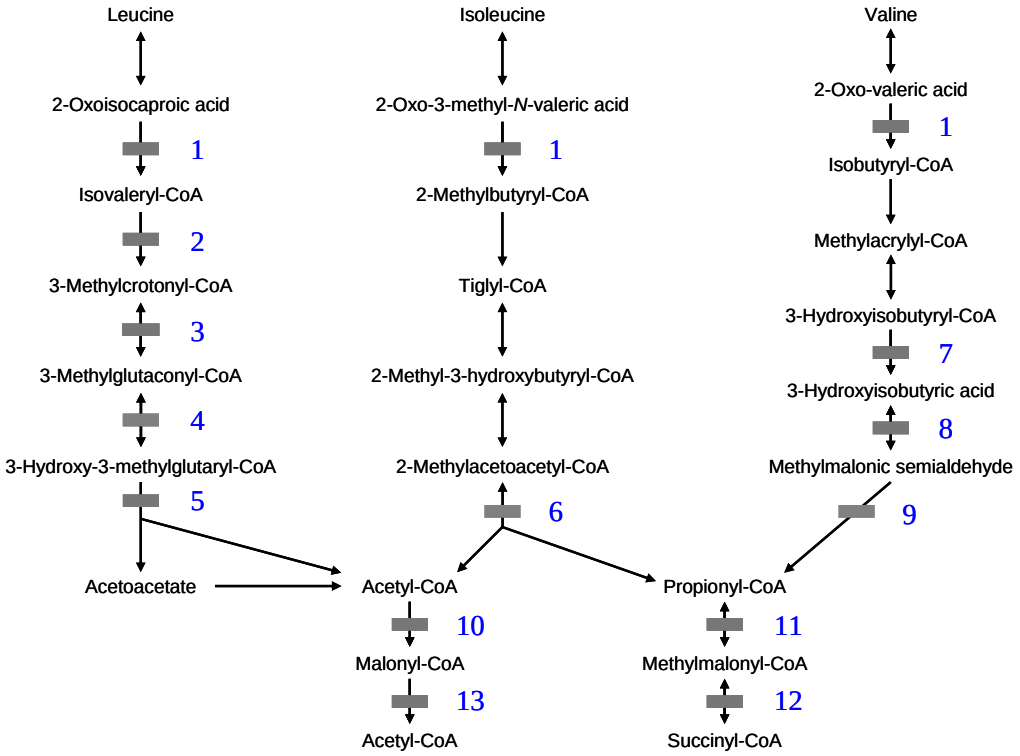


Figure 2: Degradation pathway of branched-chain amino acids and the corresponding enzymes.
(1) Branched-chain oxo-(or keto-) acid dehydrogenase; (2) isovaleryl-coenzyme A (CoA) dehydrogenase; (3) 3-methylcrotonyl-CoA carboxylase; (4) 3-methylglutaconyl-CoA hydratase; (5) 3-hydroxy-3-methylglutaryl-CoA lyase; (6) 2-methylacetoacetyl-CoA thiolase; (7) 3-hydroxyisobutyryl-CoA deacylase; (8) 3-hydroxyisobutyric dehydrogenase; (9) methylmalonylsemialdehyde dehydrogenase; (10) acetyl-CoA carboxylase (in mitochondria); (11) propionyl-CoA carboxylase; (12) methylmalonyl-CoA mutase; (13) malonyl-CoA decarboxylase. Enzyme defects are indicated by solid bars. Figure modified from (49).

2.1.1 Maple syrup urine disease (MSUD)

Deficiency of branched-chain oxo- (or keto-) acid dehydrogenase, the second step in the catabolism of the three BCAA is the cause of MSUD. The active enzyme (Figure 2, enzyme 1) is composed of three subunits (E1α, E1β and E2), a decarboxylase that requires thiamine (vitamin B_1) as a coenzyme, a dihydrolipoyl acyltransferase and a dihydrolipoamine dehydrogenase. A deficiency of any of these three subunits can cause MSUD.

MSUD is inherited as an autosomal recessive trait. The incidence is around 1:300'000. Up to date over 50 mutations have been found in the three responsible genes. They give rise to either the severe classical (75 % of all affected patients) or the intermediate phenotypes. In experimental systems gene therapy has already been tested (50).

Prenatal diagnosis relies on enzymatic assays or mutation analysis in cultured amniocytes or in chorionic villi.

Enzyme deficiency results in elevated branched-chain 2-oxo (keto-) acids in plasma, urine and cerebrospinal fluid. Due to the reversibility of the first reaction in the metabolism of BCAAs, the BCAAs also accumulate.

Together with the occurrence of the first symptoms, the patient excretes urine with an intensive maple syrup-like odor. The main laboratory finding is the presence of elevated BCAAs as well as allo-isoleucine in plasma and 2-oxo acids in the urine. Patients usually do not display severe dehydration, metabolic acidosis, accumulation of lactate or a change in blood cell count. Ammonia levels are usually normal but may be mildly elevated (<200 µmol/l).

Acutely ill newborns need emergency treatment in order to protect the brain from permanent damage. When high-energy enteral or parenteral nutrition alone is insufficient to lower plasma leucine levels, hemodialysis or hemofiltration are applied in order to lower plasma leucine levels to 1 mmol/l or less. BCAA intake has to be adjusted according to plasma levels measured daily during the time of recovery. Administration of thiamine (5 mg/kg/d) for at least three weeks has been shown to be able to improve BCAA tolerance in a few patients. Normal leucine tolerance has however never been restored (51).

The goal of the life-long therapy is to keep plasma BCAA levels near to normal concentration when measured 2-3 h after a meal. The diet is based on keeping leucine levels as normal as possible as leucine is the most toxic amino acid. Therefore, occasionally free isoleucine and valine must be added to the diet in order to provide the body requirement of those two amino acids.

Nowadays patients with MSUD are expected to survive the difficult newborn period. Generally they are healthy between times of metabolic imbalance. Some even develop normally and attend regular schools. However, during acute metabolic crisis due to intercurrent illness, patients may present with pancreatitis, epidermal lesions due to protein malnutrition or cerebral oedema that may cause unexpected death. The average intellectual performance is far below normal. Intellectual performance is inversely related to the time during which plasma leucine levels remained above 1 mmol/l and is also dependent on the long-term metabolic control.

2.1.2 Isovaleric acidemia (IVA)

Deficiency of isovaleryl-CoA dehydrogenase (Figure 2, enzyme 2), a mitochondrial flavoprotein that transfers electrons to the respiratory chain via the electron transfer flavoprotein (ETF) leads to isovaleric acidemia. Deficiency of ETF itself results in multiple acyl-CoA-dehydrogenase deficiencies (glutaric aciduria type II, (52)).

IVA is inherited as an autosomal recessive trait. The incidence is around 1:200’000. Various mutations have been described in the *IVD* gene. One mutation (p. A282V) is particularly

common in patients detected by newborn screening. They show mildly elevated metabolites and all of them have remained asymptomatic to date (53).

The defect causing IVA leads to accumulation of free isovaleric acid that is converted to 3-hydroxyisovaleric acid and N-isovalerylglycine. It can also form complexes with carnitine producing isovaleryl-carnitine. N-isovalerylglycine and 3-hydroxyisovaleric acid are rapidly excreted and are easily measured in the urine; isovaleryl-carnitine is found predominantly in the plasma.

Oral administration of L-glycine and intravenous L-carnitine are effective means of detoxification and treatment. The need for the combined supplementation is still controversial but can be useful during metabolic crisis when there is an increased need for detoxifying agents (15).

Blood exchange transfusions may be necessary for toxin removal in newborns who present in a poor clinical condition that does not allow the effective use of these alternate detoxification pathways.

Long-term therapy consists of a low protein diet supplemented with a leucine free amino acid mixture, vitamins, oral carnitine and glycine. The intake of leucine and thus natural protein can be gradually increased during the first year of life. The overall prognosis is better than for patients suffering from propionic acidemia, methylmalonic aciduria or MSUD. Intellectual outcome is dependent on early diagnosis, correct treatment and long-term compliance later in life.

2.1.3 3-Methylcrotonylglycinuria (MCC)

3-Methylcrotonylglycinuria is caused by defects in 3-methylcrotonyl-CoA carboxylase, a biotin dependent enzyme consisting of two subunits (α and β) encoded by the genes *MCCA* and *MCCB* respectively. MCC is discussed in detail in the section dealing with the biotin-dependent carboxylases (2.2.3.4).

2.1.4 Propionic acidemia (PA)

Propionic acidemia is inherited as an autosomal recessive trait and is caused by a deficiency of propionyl-CoA carboxylase, (PCC; Figure 2: enzyme 11; Figure 4: enzyme 1) one of the five biotin-dependent carboxylases found in humans. PCC is an enzyme located in the mitochondrial matrix that is composed of two non-identical subunits (α and β) encoded by the genes *PCCA* and *PCCB* respectively (54). Mutations in both genes have been found to lead to PA. PCC is involved in the catabolism of various essential amino acids like methionine, isoleucine, threonine and valine, as well as odd-chain fatty acids and cholesterol.

A deficiency results in markedly increased concentrations of free propionate in blood and urine. Major diagnostic metabolites include propionyl-carnitine in plasma, and 3-hydroxypropionate and methylcitrate in urine.

In patients with abnormal biotin metabolism, propionyl-CoA accumulation is also found due to multiple carboxylase deficiency, resulting in defective activity of all biotin-dependent carboxylases (see section 2.2.3 and Figure 7).

PA is a severe organic acidemia. This is due to the fact that the excretion of propionic acid via the urine is negligible and no alternative pathway is sufficient to detoxify the body. Patients are at high risk for life-threatening episodes of metabolic acidemia and with consecutive developmental delay, neurological symptoms and cardiomyopathy (55). During acute metabolic decompensation newborns and patients suffering from PA may thus need exogenous toxin removal procedures.

Living-donor liver transplantation has been shown to be beneficial in severely affected patients (56) but does not always prevent complications such a metabolic stroke. As a long-term treatment, gene-therapy using adeno-associated vectors is currently being investigated in mice (57). Prenatal diagnosis can be performed by mutation analysis using chorionic villi (58). For more clinical signs, diagnostic testing, therapy and prognosis see methylmalonic aciduria.

2.1.5 Methylmalonic aciduria (MMA)

Methylmalonic aciduria (MMA) is caused by a deficiency of methylmalonyl-CoA mutase (MUT; Figure 2, enzyme 12; Figure 4, enzyme 3). Methylmalonyl-CoA mutase is localized within mitochondria and catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA (Figure 3).

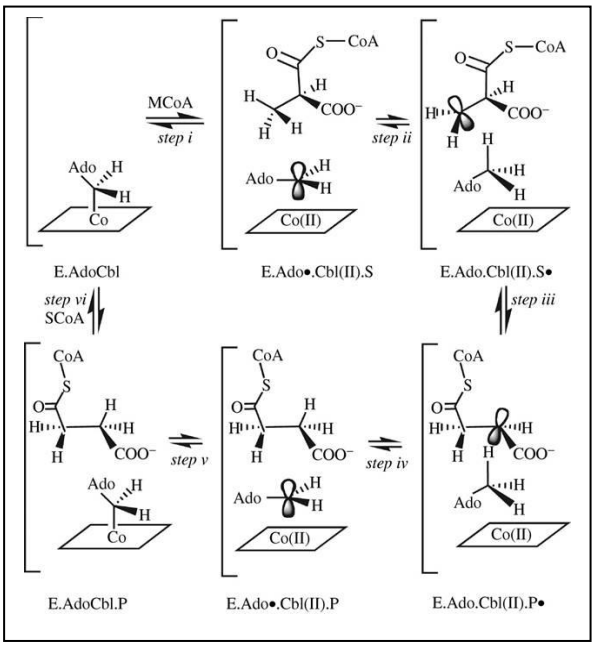


Figure 3: Methylmalonyl-CoA mutase reaction mechanism. Homolytic rupture of the metalloalkyl cobalt – carbon bond generates cob(II)alamin and the deoxyadenosyl radical (*step i*). The latter initiates the isomerization reaction by the abstraction of a hydrogen atom from the substrate to yield a substrate-centered radical (*step ii*). The substrate radical then rearranges yielding a product-centered radical (*step iii*). The product-centered radical then re-abstracts a hydrogen atom from deoxyadenosine in the second hydrogen transfer step (*step iv*). Recombination of the cofactor-based radicals completes the turnover cycle (*step v*).
MCoA and SCoA refer to methylmalonyl-CoA and succinyl-CoA, respectively.
Figure with permission from (59).

About half of the MMA patients have a mutase apoenzyme defect that is further divided into mut^0 and mut^- groups. Whereas patients with the mut^0 defect have a complete loss of enzyme activity, patients with the mut^- defect still have some residual activity and may be responsive to cobalamin (vitamin B_{12}) (60).

Various defects in intracellular cobalamin metabolism can also lead to MMA (Figure 16): while the cblF and cblC defect lead to a combination of methylmalonic aciduria and homocystinuria, the cblA and cblB defects lead to isolated methylmalonic aciduria. The cblD defect is special in that it can cause three phenotypes, isolated methylmalonic aciduria, isolated homocystinuria or a combination of both (see section 2.2.4.2).

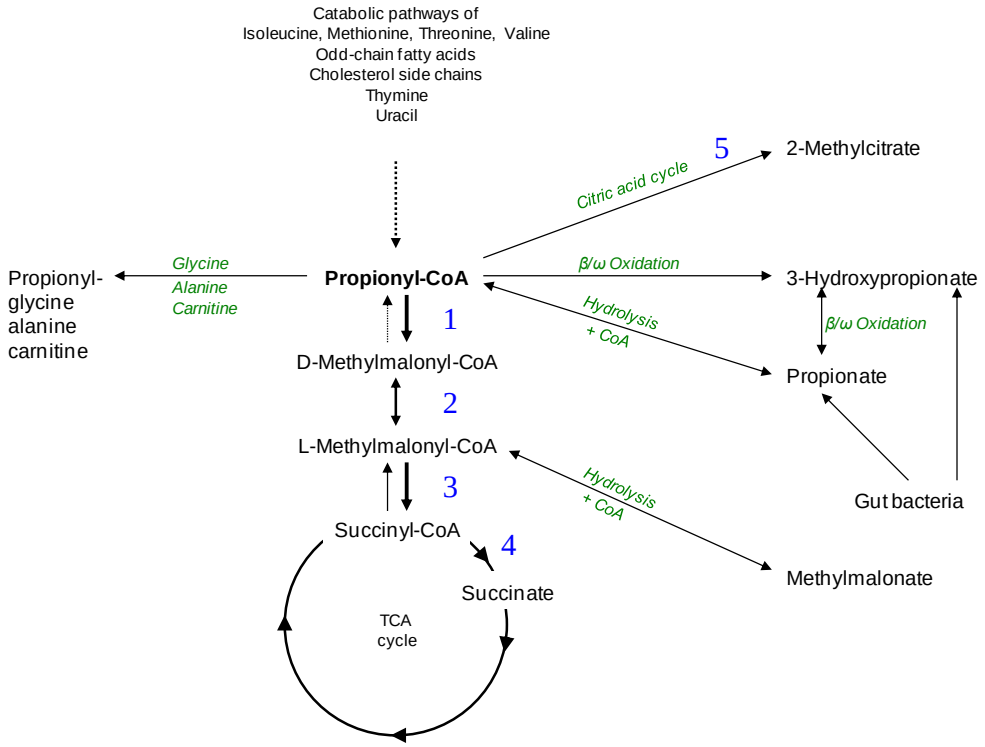


Figure 4: Metabolic pathways involved in methylmalonic aciduria. Propionyl-CoA (P-CoA), an intermediate metabolite in the common catabolic pathways of branched-chain amino acids, cholesterol, odd-numbered fatty acids, thymine and uracil, is carboxylated by propionyl-CoA carboxylase (1; reversibility of reaction is minor) to D-methylmalonyl-CoA. D-Methylmalonyl-CoA is converted to L-methylmalonyl-CoA by methylmalonyl-CoA epimerase (2, reversible reaction). Methylmalonyl-CoA mutase (MUT; 3) catalyses the conversion of L-methylmalonyl-CoA to succinyl-CoA, an intermediate of the TCA cycle. This reaction is partly reversible and is modulated by propionyl-CoA flux. Deficiency of MUT leads to alternative propionate oxidation. Propionate is derived from hydrolysis of P-CoA. β- and/or ω-oxidation of P-CoA and propionate results in formation of 3-hydroxypropionate. Gut bacteria also form propionate and 3-hydroxypropionate, which may aggravate metabolic crisis. Succinyl-CoA is converted to succinate by succinate-CoA ligase (4).

Incorporation of P-CoA instead of acetyl-CoA into the TCA cycle leads to the formation of 2-methylcitrate, catalyzed by citrate synthase (5). Other metabolites also accumulate in methylmalonic acidurias: methylmalonate is derived from hydrolysis of methylmalonyl-CoA; conjugation of P-CoA with glycine, alanine or L-carnitine produces propionylglycine, propionylalanine and propionylcarnitine. Figure modified from (61).

Mild elevations of MMA can also be found in defects of methylmalonyl-CoA epimerase (MCEE; Figure 4, enzyme 2), an enzyme converting D-methylmalonyl-CoA to L-methylmalonyl-CoA (62) or in defects of one of the various subunits of succinate-CoA ligase (SUCL; Figure 4, enzyme 4), an enzyme complex catalyzing the conversion of succinyl-CoA to succinate in the TCA cycle. Apart from these defects there are also cases of unresolved mild and benign MMA elevations (63). The total incidence of MMA is about 1:50'000.

The various defects leading to MMA are characterized by various amounts of excreted methylmalonic acid (Figure 5). As high levels of methylmalonic acid inhibit propionyl-CoA carboxylase (PCC; Figure 4, enzyme 1), a secondary accumulation of propionic acid as well as other propionyl-CoA metabolites as in PA is usually found in the urine of MMA patients.

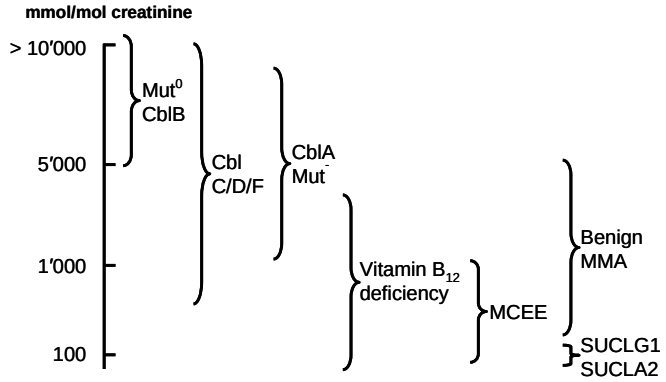


Figure 5: Urinary MMA levels in various types of disorders. The levels shown are approximations based on ranges of values reported in the literature. Mut^0 and mut^- denote a complete loss or residual activity of methylmalonyl-CoA mutase (MUT). CblA, cblB, cblC, cblD and cblF denote complementation groups of intracellular vitamin B_{12} metabolism leading to MMA. MCEE, methylmalonyl-CoA epimerase deficiency; MMA, methylmalonic aciduria; SUCLG1, succinate-CoA ligase, alpha subunit; SUCLA2, succinate-CoA ligase, ADP-forming beta subunit.
Figure from (64).

2.1.5.1 Clinical findings in methylmalonic aciduria

Accumulation of methylmalonic as well as propionic acid cause the so called "intoxication type" neurological distress with acute and rapidly progressive neurological signs (65). Infants present during the first days of life with signs of acute metabolic decompensation including vomiting, respiratory distress, feeding intolerance, hepatomegaly, hypotonia, encephalopathy, lethargy and severe acidosis. If not treated aggressively, these symptoms may progress rapidly and lead to coma and death (61, 66, 67). Acute heart failure due to cardiomyopathy, pancreatitis and epidermal lesions may also be present. In contrast to MSUD, IVA, PA and MMA patients frequently present with dehydration and moderate hepatomegaly. Also metabolic acidosis (pH<7.30) with an increased anion gap and ketonuria can be observed. Hyperammonemia is a common finding and may be very high (>800 µmol/l). In these cases it can lead to respiratory alkalosis and the disease can be misdiagnosed as a urea cycle disorder.

Neurological problems as well as renal and cardiac symptoms may present during intercurrent episodes or may progressively arise as late complications. Neuro-imaging in some MMA patients revealed symmetric degeneration of the basal ganglia; furthermore, histopathology showed severe necrosis in the globus pallidus and mild spongiosis of the subthalamic nucleus (68, 69). These pathologic findings have been suggested to be caused by "metabolic stroke" due to the accumulation of toxic organic acids (70). A recent analysis of 52 children with MMA showed that the radiological findings are unspecific, however combined incidence of common clinical and radiological findings should raise the suspicion of MMA (71).
Patients with defects in cblB or mut^0 usually present with a severe phenotype with vomiting, failure to thrive, hypotonia and encephalopathy (72) while cblA and mut^- patients usually have a milder clinical spectrum and a higher survival rate (73).

MMA in combination with homocystinuria in patients with a defect in cobalamin (vitamin B_{12}) metabolism, namely in the cblF, cblC or cblD complementation groups (see section 2.2.4.2) usually present with a different clinical picture than patients with isolated MMA (cblA and cblB).
Based on the age of onset of symptoms, 2 distinct clinical forms of the cblC defects have been recognized: The early-onset form, in which symptoms appear in the first year of life, include a multisystemic disease with severe neurological signs associated with characteristic hematological, renal and gastrointestinal manifestations. The long-term prognosis in these cases is usually poor. Patients with a later-onset form present with a milder clinical phenotype with moderate neurological involvement and behavioral disturbances (74).
Patients with the cblD defect can present with various biochemical and clinical phenotypes, namely with isolated MMA, isolated homocystinuria or the combination of these two, depending on the nature and position of the mutation within the *MMADHC* gene see chapter 2 (75, 76).
Mutations in SUCL subunits lead to mitochondrial DNA depletion; clinical symptoms include encephalomyopathy, dystonia, deafness and fatal lactic acidosis (77, 78).

2.1.5.2 Diagnosis of methylmalonic aciduria

IVA, PA and MMA are diagnosed by their specific organic acid profiles. Prenatal diagnosis can be performed by direct measurement of metabolites in amniotic fluid using various methods or by molecular genetic analysis if the mutations of the index patient are known (79, 80).
Diagnosis of MMA is based on the presence of characteristic compounds in body fluids. Methylmalonic acid, 2-methylcitric acid and 3-hydroxypropionic acid are elevated in MMA patients and can be detected by GC/MS. 2-Methylcitrate, a metabolite frequently detected in MMA and PA patients is formed by condensation of propionyl-CoA and oxaloacetate, a reaction catalyzed by citrate synthase (Figure 4, enzyme 5).

Plasma acylcarnitine analysis by MS/MS shows an abnormal profile with propionylcarnitine being the predominant carnitine ester. Furthermore the ratio of propionylcarnitine to free carnitine is greatly increased.

Due to the accumulation of propionyl-CoA in MMA and PA, ureagenesis is disturbed. This is due to propionyl-CoA-induced inhibition of N-acetylglutamate synthetase, resulting in decreased N-acetylglutamate, an allosteric activator required for carbamoylphosphate synthase I (81), and subsequently hyperammonemia.

2.1.5.3 Pathogenesis of methylmalonic aciduria

Methylcitrate exerts several inhibitory effects on enzymes of the TCA cycle and on the mitochondrial citrate transporter which facilitates mitochondrial accumulation of methylcitrate.

Impaired energy metabolism (due to dysregulation of the TCA cycle) within neurons can lead to reduced ability to maintain the normal resting membrane potential due to a depletion of ATP. This can lead to an uncontrolled influx of Na^+ leading to increase in activity of a glutamate receptor that regulates whole cell current and plays important roles in regulating plasticity and toxicity in neurons (82). Malonate, another metabolite, is only slightly elevated, it might only play a minor role in MMA-induced neurotoxicity.

It is hypothesized that accumulation of these organic acids leads to mitochondrial dysfunction. The fact that patients with inherited deficiency of mitochondrial complex II also show symmetrical lesions in the basal ganglia corroborates this hypothesis (83). Furthermore, MMA induces neuronal damage in cultured embryonic rat striatal cells at concentrations encountered in affected patients (84, 85).

Elevated ammonium levels might also be a causative agent in MMA-induced neurotoxicity (65).

The pathogenesis of acute heart failure in MMA and PA patients is still unclear (86), the same is true for renal failure; however for the latter, tubulointerstitial nephritis as well as accumulating methylmalonic acid in renal tubular cells, leading to disturbance of cellular energy metabolism, might play important roles (87).

2.1.5.4 Treatment of methylmalonic aciduria

As some forms of MMA with neonatal-onset are responsive to vitamin B_{12}, parenteral vitamin therapy, starting with 1-2 mg hydroxocobalamin per day for a few days, must be tested. B_{12} responsiveness leads to a prompt decrease in MMA and propionyl-CoA by-products. Those MMA patients responsive to B_{12} are usually characterized by a mild disease and a good outcome and require only mild protein restriction.

Otherwise the management of MMA and PA remains difficult. Treatment can be divided into emergency treatment and long-term treatment (88).

Emergency treatment is based on avoidance of nitrogen intake and the inhibition of endogenous catabolism by provision of adequate caloric intake as parenteral glucose. Insulin may be administered utilizing its anabolism-promoting effect. Metabolic acidosis is usually corrected by rehydration but may require bicarbonate therapy if pH does not normalize. Intravenous administration of carnitine is used to buffer the intramitochondrial accumulation of propionyl-CoA.

Patients unresponsive to medical therapy within the first few hours need extracorporeal removal of toxic metabolites using hemofiltration or hemodialysis (89).

Long-term treatment is based on a low-protein high-energy diet low in propiogenic substances supplemented with vitamins and trace elements.

Valine is one of the direct precursors of propionyl-CoA, therefore natural protein intake is adjusted to valine intake as the basis. Additionally, an intake of an amino acid mixture free of valine, isoleucine, methionine and threonine may be required in order to enable normal growth and prevent protein deficiency. Long-term oral administration of L-carnitine (100 mg/kg/day) seems to be effective in preventing carnitine depletion as well as enhancing urinary excretion of carnitine esters and therefore reducing propionate toxicity (90).

Another considerable source of propionate are bacteria in the intestine. Metronidazole inhibits anaerobic colonic flora and can reduce urinary excretion of propionate metabolites by up to 40 %. The recommended therapy regimen is to take metronidazole for 10 consecutive days at a dose of 10-20 mg/kg once every month (90).

Recent attempts of using liver transplantation have showed that replacement of the liver only partly corrects the enzymatic defect. Urinary metabolites are still excreted and renal failure and neurological deterioration are not cured by liver transplantation (91-93). Kidney transplantation may be necessary in the second decade of life as chronic renal failure is increasingly recognized in MMA patients, especially in those with mut^0 and cblB defects (94). For patients in end-stage renal failure, combined liver-kidney transplantation could represent a better strategy than liver transplantation alone (95).

A few reports can be found on successful pregnancies in women with MMA. Protein restriction in combination with vitamin B_{12}, carnitine and bicarbonate supplementation resulted in good outcome with birth of healthy children. Glucose infusions were used to prevent catabolism in the post-partum period (96-98).

2.2 Vitamins with a focus on biotin and cobalamin

2.2.1 Introduction

Vitamins are organic compounds that are required by an organism in small amounts and must be obtained from the diet. They are classified by their biological and chemical activity, not their

structure. Vitamins serve diverse biochemical functions, including function as hormones (e.g. vitamin D), antioxidants (e.g. vitamin E), and mediators of cell signalling and regulators of cell and tissue growth and differentiation (e.g. vitamin A). The B complex vitamins represents the largest group of vitamins functioning as coenzymes, that act as catalysts and substrates in metabolism. A list of vitamins is given in Table 1.

Name	Compound	Source	Discovered	Solubility	RDI*	Deficiency disease
Vitamin A	Retinoids	Cod liver oil	1909	Fat	900 µg	Night-blindness and Keratomalacia
Vitamin B1	Thiamine	Rice bran	1912	Water	1.2 mg	Beriberi, Wernicke-Korsakoff syndrome
Vitamin B2	Riboflavin	Eggs	1920	Water	1.3 mg	Ariboflavinosis
Vitamin B3	Niacin, niacinamide	Liver	1936	Water	16 mg	Pellagra
Vitamin B5	Pantothenic acid	Liver	1931	Water	5 mg	Paresthesia
Vitamin B6	Pyridoxine, pyridoxamine, pyridoxal	Rice bran	1934	Water	1.5 mg	Anemia
Vitamin B7	Biotin	Liver	1931	Water	30 µg	Dermatitis, enteritis
Vitamin B9	Folic acid, folinic acid	Liver	1941	Water	400 µg	Deficiency during pregnancy is associated with birth defects, such as neural tube defects
Vitamin B12	various Cobalamins	Liver	1926	Water	2.4 µg	Megaloblastic anemia
Vitamin C	Ascorbic acid	Lemons	1912	Water	90.0 mg	Scurvy
Vitamin D	Ergocalciferol, cholecalciferol	Cod liver oil	1918	Fat	10 µg	Rickets and Osteomalacia
Vitamin E	Tocopherols, tocotrienols	Wheat germ oil and liver	1922	Fat	15 mg	Deficiency is very rare; mild hemolytic anemia in newborn infants
Vitamin K	phylloquinone, menaquinones	Alfalfa	1929	Fat	120 µg	Bleeding diathesis

*: recommended dietary intake

Table 1: list of vitamins and their properties.

Deficiencies of vitamins are classified as either primary or secondary. A primary deficiency refers to a state in which an organism does not acquire enough of the vitamin with the diet. A secondary deficiency may be due to an underlying disorder that prevents or limits the absorption or use of the vitamin. This is most likely due to a “lifestyle factor” such as smoking, exposure to sunlight, excessive alcohol consumption and many more, or more interesting to researchers - due to a genetic defect that interferes with the absorption or use of the vitamin.

People consuming a varied diet are unlikely to develop a severe primary vitamin deficiency. In contrast, restrictive diets caused by vegetarianism, food allergies, poor availability of certain food stuffs, malnutrition in general and many more, have the potential to cause prolonged vitamin deficits, which may result in potentially fatal diseases (99-102).

2.2.2 Biotin (vitamin B_7)

Biotin was discovered around 1900 when it was shown to be essential for the growth of certain yeast strains. It was not until 1943 when the chemical formula (Figure 6) was elucidated (103). In humans biotin is necessary for cell growth, the formation of fatty acids and the metabolism of fats and amino acids. Biotin-containing foods include berries, cereals, liver, milk, nuts, vegetables and yeast.

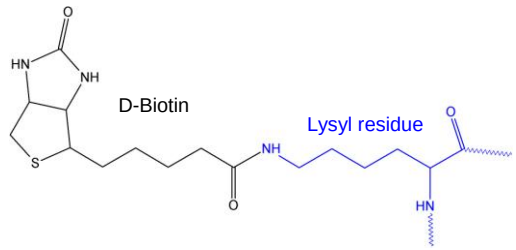


Figure 6: Structure of biotin In this sketch D-biotin is bound to a protein via a lysyl residue.

Biotin is taken up in the diet in the free as well as the protein-bound form. The enzyme biotinidase releases the protein-bound biotin and makes it available for intestinal uptake. Biotin is taken up in the small and to some extent also in the large intestine by carrier-mediated processes (104). After successful uptake it enters the bloodstream and is taken up by target cells again by carrier-mediated processes (105).

2.2.2.1 Biotin deficiency

Biotin deficiency can lead to seizures, hypotonia, ataxia, dermatitis, hair loss, mental retardation, ketoacidosis and organic aciduria (106). In the US, it was shown that about half of the pregnant women are marginally biotin deficient (107). While in humans this does not seem to have a great impact, in animal models this situation has been shown to be teratogenic (108). Recently, connections between biotin and gene expression patterns caught attention as various histones, protein components of chromatin, are biotinylated (109). In animal models it was shown that a decrease in histone biotinylation caused abnormal gene expression and reduced life-span as well as heat resistance (110). The mechanisms whereby histone biotinylation leads to gene repression still need to be elucidated and the extent to which biotinylation of histones alters gene expression in humans warrants further investigation (107).

2.2.3 Biotin-dependent carboxylases and intracellular biotin metabolism

In humans, five biotin-dependent carboxylases have been described so far (Table 2): acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2), 3-methylcrotonyl-CoA carboxylase (MCC), propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (PC) (106, 111).

	ACC1	ACC2	MCC		PC	PCC	
location	cytosolic	mitochondria	mitochondria		mitochondria	mitochondria	
subunits	1	2	α	β	α	α	β
structure	polymeric?	polymeric?	$(\alpha\beta)_6$		α_4	$(\alpha\beta)_6$	
amino acids	2383	2483	725	563	1178	703	539
size (kDa)	269	279.7	80.5	61.3	129.6	77.4	58.2
exons	54	48	19	17	21	24	15
locus	17q21	12q24.1	3q25-27	5q12-13	11q13.3	13q32	3q21-q22

Table 2: Table of the five biotin-dependent carboxylases found in humans indicating their localization, hypothetical structure, size and genetic organization

Owing to the role of the carboxylases in various metabolic pathways like gluconeogenesis, fatty acid synthesis and the catabolism of several amino acids (Figure 7), their deficiency may lead to profound metabolic derangement, eliciting neurological symptoms and a characteristic organic aciduria (106).

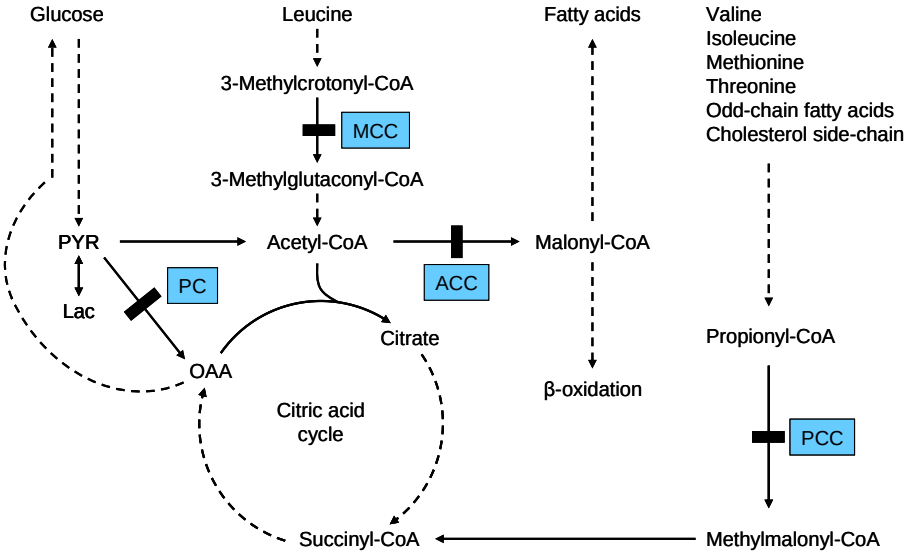


Figure 7: The carboxylase deficiencies. Sites of enzyme defects are indicated by black bars. Full lines indicate one enzyme and dashed lines indicate that several enzymes are involved. Pyr, pyruvate; lac, lactate; OAA, oxaloacetate; MCC, 3-methylcrotonyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; PC, pyruvate carboxylase; ACC, acetyl-CoA carboxylase. Figure modified with permission from (112).

The five biotin-dependent carboxylases all have three structurally conserved functional domains: the biotin carboxyl carrier domain, which carries the biotin prosthetic group; the biotin carboxylation domain, which catalyzes the carboxylation of biotin and the carboxyltransferase domain, which catalyzes the transfer of a carboxyl group from carboxybiotin to the organic substrate specific for each carboxylase (113). The genes for all human carboxylases have been cloned and characterized (54, 114-116).

The intracellular formation of the active carboxylase (holocarboxylase) occurs in two partial reactions: After the formation of the biotinyl-5'-AMP intermediate, the biotinyl moiety is transferred to the apoenzyme, forming the active holoenzyme:

A) d-biotin + ATP $\overset{Mg^{2+}}{\rightleftharpoons}$ d-biotinyl-5'AMP + PP_i

B) d-biotinyl-5'AMP + apocarboxylase $\rightleftharpoons$ holocarboxylase + AMP

Net: d-biotin + ATP + apocarboxylase $\overset{Mg^{2+}}{\rightarrow}$ holocarboxylase + AMP + PP_i

The action of biotin-dependent enzymes can be expressed as two partial reactions: The first reaction involves the formation of the carboxybiotinyl enzyme using bicarbonate as the carboxyl donor. In the second reaction, the carboxyl group is then transferred from the enzyme-CO_2 complex to an acceptor substrate. This mechanism has been shown for all five biotin-dependent carboxylases (117).

C) Enzyme-biotin + ATP + HCO_3^- $\overset{Mg^{2+}}{\rightleftharpoons}$ Enzyme-biotin-CO_2 + ADP + PP_i

D) Enzyme-biotin-CO_2 + acceptor $\rightleftharpoons$ Enzyme-biotin + acceptor-CO_2

Net: HCO_3^- + ATP + acceptor $\overset{Mg^{2+}}{\rightleftharpoons}$ acceptor-CO_2 + ADP + PP_i

The active holocarboxylases are degraded by proteases during the course of normal cellular turnover. End-products of this degradation are free amino acids and the small peptide biocytin (biotinyl-lysine). The final release of biotin from biocytin is again carried out by biotinidase (Figure 8). Deficiency of either biotinidase or holocarboxylase synthetase results in multiple carboxylase deficiency (118-121).

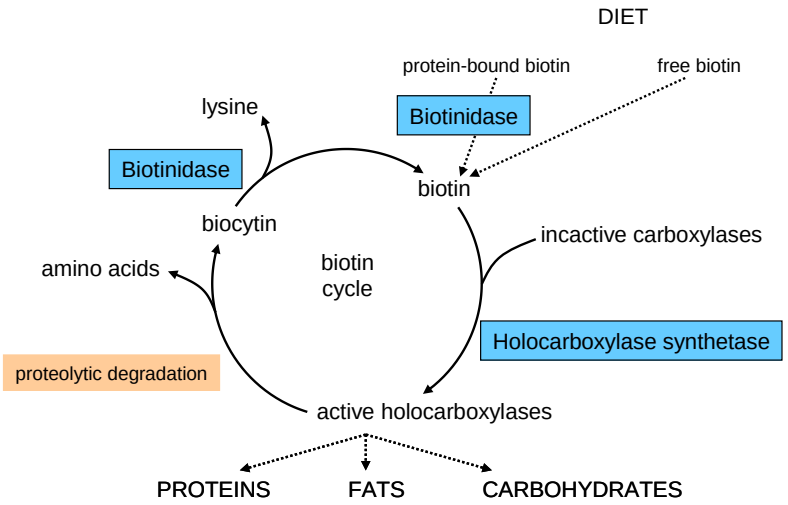


Figure 8: The intracellular biotin cycle. Biotin is taken up by the diet either in the free or protein-bound form. Biotinidase releases free biotin from protein-bound biotin. Holocarboxylase synthetase attaches free biotin to inactive carboxylases producing active holocarboxylases. These are important in the synthesis and degradation of proteins, amino acids, fats and carbohydrates. Proteolytic degradation of active holocarboxylases leads to the release of amino acids and biocytin (biotinyl-lysine). Biotinidase cleaves biocytin thereby releasing free biotin.

2.2.3.1 Acetyl-CoA carboxylase (ACC1 and ACC2, EC 6.4.1.2)

In humans there are two ACC genes, *ACC1* and *ACC2* (115). While *ACC1* is mainly expressed in lipogenic tissues, *ACC2* is predominantly found to be expressed in muscle and heart (122-124). While ACC1 appears to be localized in the cytosol where it is a major

provider of malonyl-CoA that is needed for the synthesis of very long-chain fatty acids, ACC2 seems to be localized at the mitochondrial membrane and is thought to be responsible for mitochondrial fatty acid oxidation (125, 126). As ACC plays pivotal roles in intracellular energy metabolism and has been linked to various diseases like cancer, diabetes and microbial infections (127, 128), it has become an attractive drug target (129).
In mice it has been shown that animals lacking ACC1 are embryonically lethal (126). In humans, deficiency of ACC has only been reported in one patient, a newborn girl that presented with severe respiratory and perinatal problems as well as hypotonic myopathy. Later it became clear that she had severe brain damage, persistent myopathy and poor growth (130).

2.2.3.2 Pyruvate carboxylase (PC, EC 6.4.1.1)

PC is a key enzyme in gluconeogenesis and is located in the mitochondrial matrix. It is mainly expressed in gluconeogenic tissue where it catalyzes the first step in the synthesis of glucose from pyruvate. It is also expressed in lipogenic tissue where it participates in the formation of acetyl groups and reducing groups for transport from mitochondria to the cytosol (114). It has been suggested that PC is also involved in regulating glucose-induced insulin release (131, 132).
Individuals suffering from PC deficiency exhibit a variety of clinical and biochemical abnormalities including metabolic acidosis, hypotonia, delayed physical and mental development and seizures. Those who survive are severely retarded (114, 117, 133).
Currently the recommended dietary treatment for PC deficiency offers limited options since a high-fat diet would increase ketoacidosis, while a high carbohydrate diet would exacerbate lactic acidosis (134).

2.2.3.3 Propionyl-CoA carboxylase (PCC, EC 6.4.1.3)

See section 2.1.4 for information about PCC and the effects of PCC deficiency.

2.2.3.4 3-Methylcrotonyl-CoA carboxylase (MCC, EC 6.4.1.4)

MCC catalyzes the fourth step of the leucine catabolic pathway (Figure 2, enzyme 3) by carboxylating 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA (Figure 9) in a reversible ATP-dependent reaction using bicarbonate as the source of the carboxyl group (65). MCC consists of a larger α subunit, which covalently binds biotin, and a smaller β subunit (54). The *MCCA* and *MCCB* structural genes have been mapped to 3q25-q27 and 5q12-q13.1 and have 19 and 17 exons, and have a size of 80 and 61 kDa, respectively (116, 135, 136). MCC is predominantly localized to the inner membrane of mitochondria and is highly expressed in

kidney and liver (65). The α and β subunits are synthesized in the cytoplasm and are transported into the mitochondria by means of a signal peptide (137, 138).

Recently, the first recombinant baculovirus expressed MCC has been generated, purified as a 500-800 kDa complex and found to be enzymatically active with enzyme characteristics similar to those reported for the native enzyme (139). Bovine MCC has a similar size and has been shown to be comprised of six heterodimers (140).

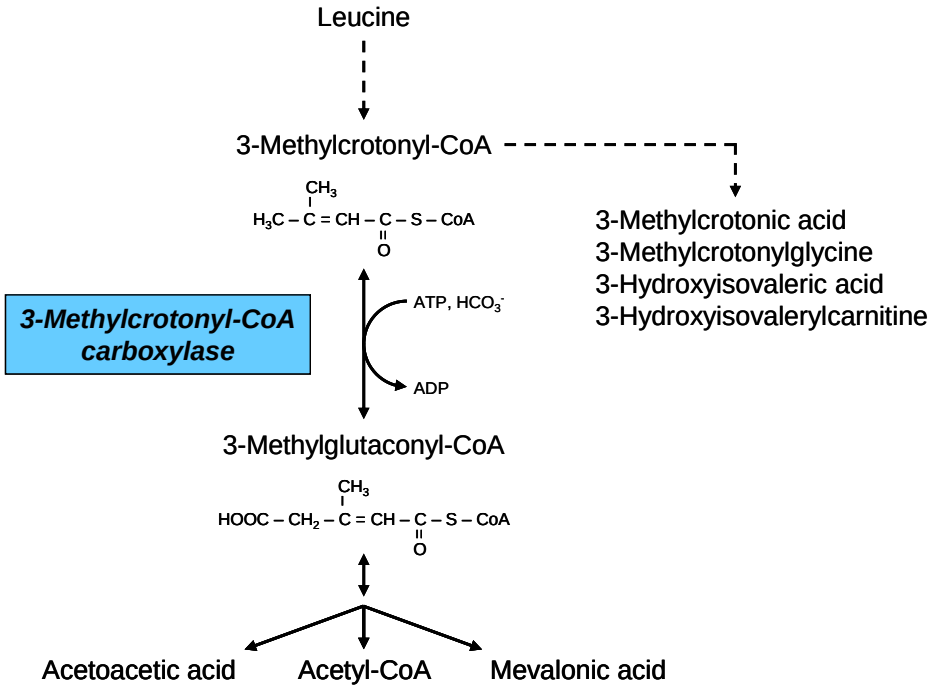


Figure 9: Degradation pathway of leucine. 3-Methylcrotonyl-CoA is converted to 3-methylglutaconyl-CoA by MCC in an ATP and bicarbonate dependent reaction. MCC deficiency leads to the accumulation of abnormal metabolites, like 3-methylcrotonic acid, 3-methylcrotonylglycine, 3-hydroxyisovaleric acid and 3-hydroxyisovalerylcarnitine. ATP, adenosine triphosphate; ADP, adenosine diphosphate; HCO_3^-, bicarbonate; CoA, coenzyme A (modified from (116)).

2.2.3.4.1 3-Methylcrotonyl-CoA carboxylase deficiency (MIM 210200 and 210210)

MCC deficiency is caused either by defects of the MCC enzyme itself or by deficient activity of the enzymes involved in the metabolism of its cofactor biotin, i.e. holocarboxylase synthetase (HCS) or biotinidase, which cause multiple carboxylase deficiency. Isolated biotin-resistant MCC deficiency (also known as methylcrotonylglycinuria) is inherited as an autosomal recessive trait (65). The disorder must be distinguished from the biotin-responsive multiple carboxylase deficiencies. In isolated MCC deficiency, increased urinary levels of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine are characteristically found. In multiple carboxylase deficiencies additional metabolites which are characteristic of PC and PCC deficiencies, e.g. lactic acid, 3-hydroxypropionate and small amounts of methylcitrate are

present. Confirmation of the isolated deficiency requires an activity assay of MCC and at least one other carboxylase, usually PCC.

The first case of MCC deficiency was described in a 4.5 months old girl, from consanguineous background, that had elevated urinary excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. However, no enzyme activities were measured, and the child died in spite of being on a low-leucine diet (141, 142).

Over 45 cases of isolated MCC deficiency have been clinically described (143-149). Most patients are asymptomatic until an episode of acute metabolic crisis following intercurrent illness or introduction of a protein-rich diet in early childhood leads to their diagnosis (65). Patients presenting with severe symptoms are commonly from consanguineous marriages, the clinical symptoms may therefore be due to a different, yet unknown primary disorder.

Symptoms can include vomiting, opisthotonus, involuntary movements, seizures, muscular hypotonia, coma and apnoea. Typical laboratory findings during an acute episode include severe hypoglycemia, hyperammonemia, elevated hepatic transaminases, mild metabolic acidosis and moderate ketonuria. Patients with MCC deficiency show a persistently high urinary excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine, usually in combination with a severe secondary carnitine deficiency. In addition, acyl-CoA derivatives accumulate and are esterified to acylcarnitine esters. The major abnormal metabolite found in blood and urine is 3-hydroxyisovalerylcarnitine (65).

Patients usually respond to intravenous fluids and cessation of protein feeding and are asymptomatic between acute episodes. Some affected subjects have been placed on a modestly leucine restricted diet supplemented with oral L-carnitine and glycine to enhance detoxification ability of the accumulating toxic metabolites. It seems that the glycine and L-carnitine detoxification systems are complementary to each other rather than competitive (150-153). No comprehensive studies of intellectual and/or developmental outcome have been published so far.

MCC activity in homogenates of cultured skin fibroblasts of patients is usually less than 2 % of control values, although 4 patients with activity from 4-12 % of normal have been reported (116, 148, 154). To date, many functionally significant mutations in both *MCCA* and *MCCB* have been reported including missense, nonsense, frame-shift and splice-site mutations (116, 135, 136, 149, 155, 156).

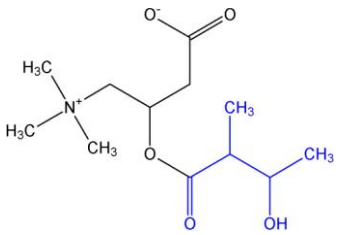
So far, no genotype-phenotype correlation has been found. MCCB E99Q for example was detected in a boy that died (157) but also in an adult woman that was asymptomatic (158). The same is true for other mutations that have been described in both severe and asymptomatic cases (146, 156, 159, 160). Also no correlation between the level of residual enzyme activity and clinical presentation has been observed.

Before the advent of tandem mass spectrometry (TMS) technology and the introduction of newborn screening (NBS) programs in the late 1990s, it was thought that MCC deficiency was an extremely rare metabolic disorder. However, the introduction of TMS based NBS programs in North America (158, 161-163), Europe (146, 164, 165) and Australia (42) showed that MCC deficiency is the most frequent organic aciduria detected with an overall frequency of around 1:50'0000. Comparative analysis of case reports with NBS data implied that very few individuals (<10 %) develop symptoms (146). This together with a number of affected siblings of symptomatic patients that have been clinically normal (166-168), suggests that the genotype at the MCC locus is not the sole determining factor in the manifestation of the disease and that MCC deficiency represents a complex monogenetic trait (65) with a functionally mild expression and low clinical penetrance.

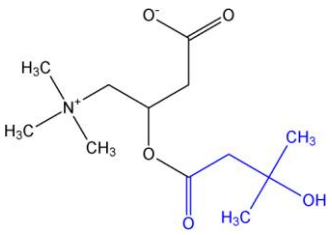
Furthermore, the clinical phenotype is highly variable: some patients present in the neonatal period with one or more of the above mentioned symptoms and have a poor outcome despite treatment (157, 169, 170). Others are asymptomatic siblings and adults detected in family studies or due to positive newborn screening findings in their unaffected babies (146, 158, 161). A similar finding can be made in isovaleric acidemia (IVA, section 2.1.2), this disease is caused by mutations in the gene coding for isovaleryl-CoA dehydrogenase. The clinical penetrance of IVA is much greater than for MCC deficiency however there is also a mutation often detected by NBS that leads to a biochemical phenotype (excretion of abnormal metabolites) but does not produce a clinical phenotype.

2.2.3.4.2 Newborn Screening and 3-methylcrotonyl-CoA carboxylase deficiency

The marker for screening for MCC deficiency is 3-hydroxyisovalerylcarnitine, a member of the C5OH carnitines. Another C5OH found in humans is 2-methyl-3-hydroxybutyrylcarnitine. These isomeric carnitines (Figure 10) can only be distinguished by HPLC/MS, not by MS/MS (171-173). As MS/MS is the standard method used for NBS, elevated levels of C5OH can be found in patients having various defects including 3-methylcrotonyl-CoA carboxylase deficiency, 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, 2-methylacetoacetyl-CoA thiolase deficiency, 3-methylglutaconyl-CoA hydratase deficiency as well as biotinidase deficiency, holocarboxylase deficiency and dietary biotin deficiency (174). Screening for C5OH may be warranted when considering the wide range of significant conditions that can be identified. However there seems to be a need for a proper research study evaluating the benefits of screening for this metabolite (Bridget Wilcken, personal communication).



3-Hydroxyisovalerylcarnitine



2-Methyl-3-hydroxybutyrylcarnitine

Figure 10: two isomeric C5OH carnitines found in various diseases. These two isomeric carnitines can only be distinguished by HPLC/MS and not by MS/MS, the method used in routine NBS programs.

Currently MCC deficiency is still part of expanded NBS programs in Australia, Austria, Canada, Denmark, the Netherlands, New Zealand and the US (40, 175, 176). However, controversy is starting to mount around the continued inclusion of MCC deficiency in NBS programs. A recent analysis of NBS in Bavaria in 677'852 neonates revealed 8 newborns and 6 relatives with MCC deficiency (146). The individuals were all asymptomatic or had benign phenotypes. Calculated risk of death has been estimated to be at maximum 1:8'500'000, the risk of clinical symptoms 1:1'200'000 (146).

Up to now it is impossible to stratify risk precisely in babies diagnosed for MCC deficiency by NBS to suffer from clinical symptoms, however it is important to adopt appropriate management protocols and not to over-react and cause more harm than good (177, 178). In Germany the low ratio of benefit to harm has resulted in the decision to exclude MCC deficiency from NBS in most federal states. Therefore, MCC deficiency is an example of the ongoing debate arising from the inclusion of potentially asymptomatic conditions, which generates a psychological burden for afflicted families and a financial burden for health care systems (41, 146, 156).

In the past, several disorders were screened for and treated but were found to be benign after all. Examples include Histidinemia (41, 179-183) or Hydroxyprolinemia (41, 184-187).

2.2.3.4.3 Aims of the 3-methylcrotonyl-CoA carboxylase project

When I started my PhD, the aim was to investigate the size of the active human MCC protein, mitochondrial import of the subunits and to describe interesting mutations in detail.

The size of the active human MCC complex has been published by Chu et al. (139) and mitochondrial import of the subunits has been investigated by Stadler et al. (138). Therefore we focused on describing an interesting mutation we found in one of our MCC patients.

2.2.4 Cobalamin (vitamin B_{12})

Vitamin B_{12} (cobalamin) is the largest and chemically most complex of all the vitamins (Figure 11). It is thought to have originated some 3 billion years ago when no oxygen was present yet and other molecules carried out oxidation reactions. The metal-ions in enzymes carrying out these reactions included nickel, iron, manganese and cobalt. With the advent of oxygen, sulphur was oxidized and subsequently formed insoluble complexes with these metal ions. As a result many enzymes replaced their metal-ions by zinc or copper. Cobalamin can therefore be regarded as a legacy of ancient times (188).

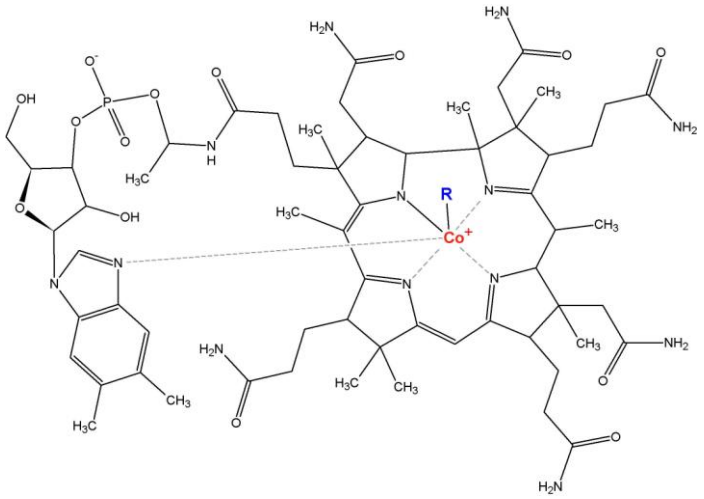


Figure 11: Structure of cobalamin (vitamin B_{12}). The cobalt ion is in the middle of a corrin ring, R represents the various side-chains (Methyl-, 5'-Deoxyadenosyl-, Hydroxy-, Cyano- etc.).

Nowadays, cobalamin is synthesized only by certain bacteria (189, 190) and is subsequently concentrated mainly in the bodies of higher organisms. The main dietary sources of the vitamin are therefore of animal origin. Some plant foods like edible algae (e.g. *Porphyra* sp. commonly known as nori) also contain large amounts of cobalamin. For a long time the source of algal cobalamin remained a matter of controversy, however, it has been shown that algae do not synthesize it themselves, they acquire it through a symbiotic relationship with bacteria (191, 192). Whereas the algal cobalamin seems to be bioavailable to mammals, the one produced by certain cyanobacteria like *Spirulina* seems to be inactive in mammals (193). Due to the limited bioavailability, the uptake and recycling of cobalamin is highly efficient. The recommended daily allowance (RDA) is the lowest of all vitamins and is set at 2.4 µg/day in adults (194). It is important to note that during cooking or microwave heating, considerable amounts of cobalamin are destroyed (195). The same is true during fermentation processes (e.g. in the production of yoghurt and cheese from milk) (196).

Once taken up by the body, cobalamin is accumulated and stored in the liver; therefore an adult human's diet may be cobalamin deficient for many months to years before developing clinical symptoms.

In mammals, cobalamin functions as coenzyme for two enzymes. In the form of methylcobalamin (MeCbl) for the cytosolic methionine synthase (MS; EC 2.1.1.13, see section 2.2.4.1) and in the form of 5'-deoxyadenosylcobalamin (adenosylcobalamin, AdoCbl) for the mitochondrial methylmalonyl-CoA mutase (EC 5.4.99.2).
Recent findings suggest that cobalamin in the form of thiolatocobalamins may act as an intracellular antioxidant and may therefore play an important role in cell survival during inflammation (197).

2.2.4.1 Cobalamin uptake and metabolism

Dietary cobalamin enters the stomach mostly bound to animal proteins. Pepsin and hydrochloric acid cleave the animal protein, thus releasing free cobalamin. Most of the free cobalamin is subsequently bound to R-protein, a protein released from the salivary glands and the parietal cells in the stomach. Another protein, "intrinsic factor" is also secreted by the stomach; however, its binding to cobalamin is weak in the presence of R-protein. In the duodenum, pancreatic enzymes degrade the cobalamin-R-protein complexes releasing free cobalamin. This is when cobalamin binds to intrinsic factor. The resulting cobalamin-intrinsic factor complex remains undisturbed until the distal part of the ileum where it is taken up by epithelial cells via the intrinsic factor receptor complex, containing the proteins cubilin and amnionless, through receptor-mediated endocytosis. Within the epithelial cells, cobalamin is bound to transcobalamin I & II (transcobalamin III has been shown to be identical to transcobalamin I, the only difference is the charge, presumably due to a different glycosylation pattern; Ralph Carmel, personal communication), these complexes enter the bloodstream and are distributed throughout the body (Figure 12).
More than 70 % of the cobalamin in the blood circulates bound to transcobalamin I, a protein that has been shown to be identical with R-protein, the function of this complex is still unknown (198). On the plasma membrane of target cells the cobalamin-transcobalamin II complex binds to the transcobalamin receptor (Figure 16; TCblR) and is internalized by receptor-mediated endocytosis (199, 200).

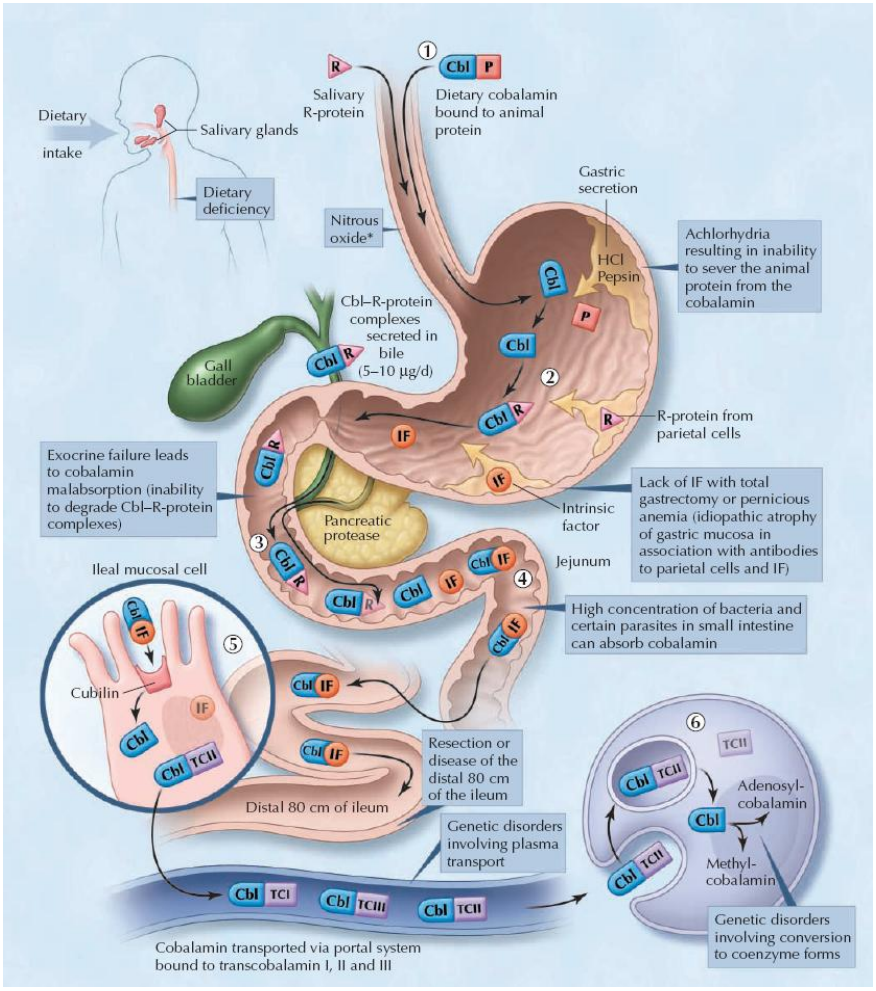


Figure 12: Cobalamin metabolism and corresponding causes of deficiency.

Causes of cobalamin deficiency are shown in blue. The metabolic pathway starts when (1) dietary cobalamin (Cbl), obtained through animal foods, enters the stomach bound to animal proteins (P). (2) Pepsin and hydrochloric acid (HCl) in the stomach sever the animal protein, releasing free cobalamin. Most of the free cobalamin is then bound to R-protein (R), which is released from the salivary and parietal cells. Intrinsic factor (IF) is also secreted in the stomach, but its binding to cobalamin is weak in the presence of R-protein. (3) In the duodenum, dietary cobalamin bound to R-protein is joined by cobalamin-R-protein complexes that have been secreted in the bile. Pancreatic enzymes degrade both biliary and dietary cobalamin-R-protein complexes, releasing free cobalamin. (4) Cobalamin then binds to intrinsic factor. The cobalamin-intrinsic factor complex remains undisturbed until the distal 80 cm of the ileum, where (5) it attaches to mucosal cell receptors (cubilin) and is taken up. Inside the cell cobalamin is bound to transcobalamin I-III (TCI-TCIII). Transcobalamin II, although it represents only a small fraction (about 10 %) of the transcobalamins, is the most important as it is able to deliver cobalamin to all cells in the body. The cobalamin is subsequently transported systemically via the portal system. (6) Within each cell, the transcobalamin II-cobalamin complex is taken up by means of endocytosis and the cobalamin is liberated and then converted enzymatically into its 2 coenzyme forms, methylcobalamin and adenosylcobalamin (this process is shown in greater detail in Figure 16).

*Nitrous oxide, a general anesthetic, causes multiple defects in cobalamin use, most of which are intracellular and clinically relevant only in people who have low or borderline-low serum cobalamin levels (201, 202).

It has been shown that cubilin forms a complex with amnionless in order to import cobalamin (204).

Further it was shown that TCI and TCIII are the same protein differing only in the glycosylation pattern.

Once inside the cell, cobalamin is processed by various steps leading to the formation of methylcobalamin (MeCbl) and 5'-deoxyadenosylcobalamin, referred to as adenosylcobalamin (AdoCbl) (Figure 16).

Methylcobalamin, is a cofactor for methionine synthase (MS, EC 2.1.1.13), a cytosolic enzyme that catalyzes the conversion of homocysteine to methionine (Figure 13). The reaction requires N^5-methyltetrahydrofolate and is important for the supply of tetrahydrofolate, needed for the synthesis of RNA and DNA; the synthesis of S-adenosylmethionine, required for a multitude of methylation reactions; and for the catabolism of the toxic amino acid homocysteine. Methionine synthase is composed of four distinct domains, a cobalamin binding domain, a homocysteine binding domain, a C-terminally located activation domain and tetrahydrofolate binding domain.

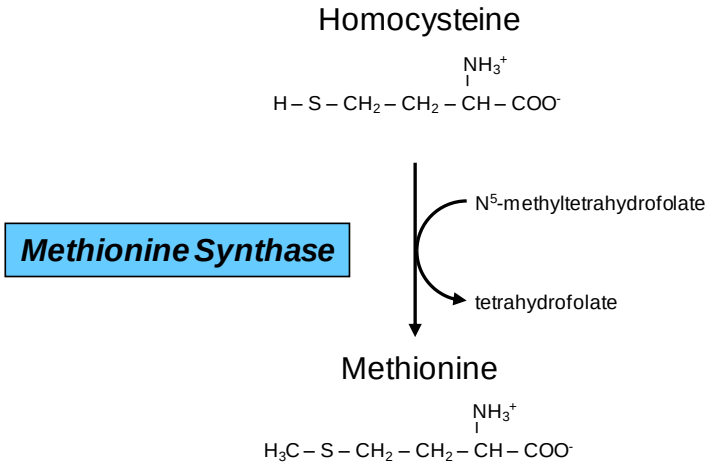


Figure 13: Degradation pathway of Homocysteine. Methionine synthase converts homocysteine to methionine in a cobalamin dependent reaction using N^5-tetrahydrofolate as a methyl-donor.

MS utilizes enzyme-bound cob(I)alamin to abstract a methyl group from N^5-methyltetrahydrofolate to generate tetrahydrofolate and methylcob(III)alamin. The methyl group is subsequently transferred to the thiol group of homocysteine to produce methionine and regenerate cob(I)alamin. The detailed reaction is depicted in Figure 14.

A) cob(I)alamin + N^5-methyltetrahydrofolate ⟶ methylcob(III)alamin + tetrahydrofolate

B) methylcob(III)alamin + homocysteine ⟶ cob(I)alamin + methionine

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Net: homocysteine + N^5-methyltetrahydrofolate ⟶ methionine + tetrahydrofolate

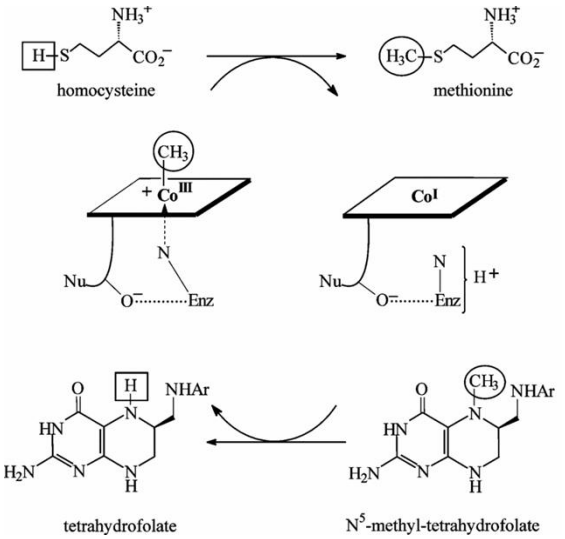

Figure 14: Detailed reaction scheme of the action of methionine synthase. Reproduced with permission from (205), www.biochemsoctrans.org.

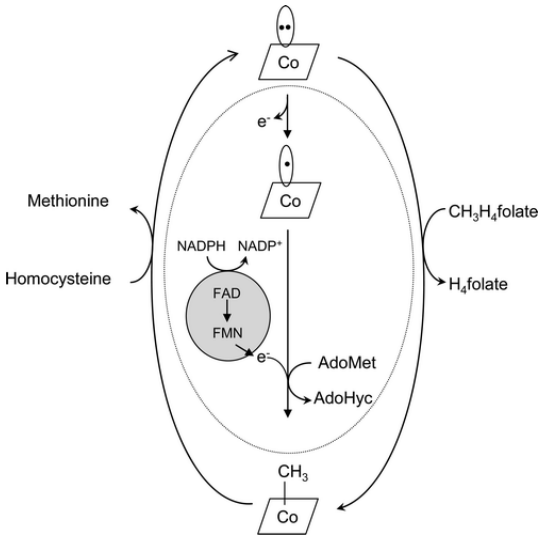

Figure 15: The catalytic cycle and reactivation of methionine synthase by methionine reductase (grey). With permission from (206).

To some extent cob(I)alamin is oxidized to cob(II)alamin, rendering MS inactive. In this case reactivation of MS requires reductive methylation of cob(II)alamin, a process involving methionine synthase reductase (MSR) and methyl transfer from S-adenosylmethionine. Reducing equivalents for the reduction of cob(II)alamin catalyzed by MSR are derived from NADPH oxidation and are delivered via the flavin adenine dinucleotide and flavin mononucleotide redox centers of MSR (Figure 15)

The second cobalamin-containing coenzyme adenosylcobalamin is a cofactor for methylmalonyl-CoA mutase (MUT), an enzyme localized within mitochondria. MUT is essential for the conversion of methylmalonyl-CoA to succinyl-CoA in the degradation of various BCAA, odd-chain fatty acids, cholesterol as well as thymine and uracil (Figure 4, enzyme 3). This reaction is described in more detail in section 2.1.

2.2.4.2 Inherited and acquired cobalamin deficiencies

Vitamin B_{12} deficiency can be caused by primary factors such as veganism or by a multitude of genetic disorders (207). The latter can be due to defects in absorption, transport, cellular uptake and intracellular processing of cobalamin.

Absorption defects include defects in intrinsic factor (203, 208), R-protein (209) or cubilin (210). Recently the proteins amnionless and megalin were reported to be associated with cubilin and to be involved in cobalamin uptake (211-214). Transport defects include defects in transcobalamin I (215-217), transcobalamin II (218-220). Cellular uptake occurs via receptor-mediated endocytosis by the transcobalamin-receptor (200).

Intrinsic factor deficiency and Imerslund-Gräsbeck syndrome (deficiency in cubilin or amnionless) have been shown to cause cobalamin deficiency in children and lead to megaloblastic anemia that may end up fatal if untreated (221). In elderly patients, cobalamin deficiency can be caused by pernicious anemia, an autoimmune disease characterized by the destruction of the gastric mucosa (222). Under these circumstances, gastric secretions are neutral to slightly acidic and contain little or no intrinsic factor (223). Pernicious anemia is further associated with many autoimmune disorders like vitiligo or Sjögren's syndrome (224).
Transcobalamin I deficiency has been shown to be difficult to distinguish from cobalamin deficiency. Furthermore, affected patients have normal methylmalonic acid and homocysteine levels, making lifelong cobalamin treatment unnecessary (198).
Deficiency of transcobalamin II can lead to fatality in early infancy but can be treated with parenteral cobalamin. Diagnosis can be difficult as plasma cobalamin levels are normal because most of the cobalamin is bound to transcobalamin I. Symptoms include megaloblastic anemia, pancytopenia, failure to thrive and eventually neurological symptoms (225, 226).
So far no patients with defects in the transcobalamin-receptor have been reported, however the phenotype would most likely share similarities with transcobalamin II deficiency.
Defects in uptake and transport of cobalamin are expected to also cause homocystinuria, however this finding is not explicitly mentioned in the above mentioned publications. It has even been shown that in some patients with a severe transcobalamin II deficiency, plasma homocysteine and amino acid levels were within normal limits (226).

To date, intracellular defects of cobalamin metabolism are divided into eight different complementation groups, each of which corresponds to a defect in one gene (Figure 16) (75, 227-234).

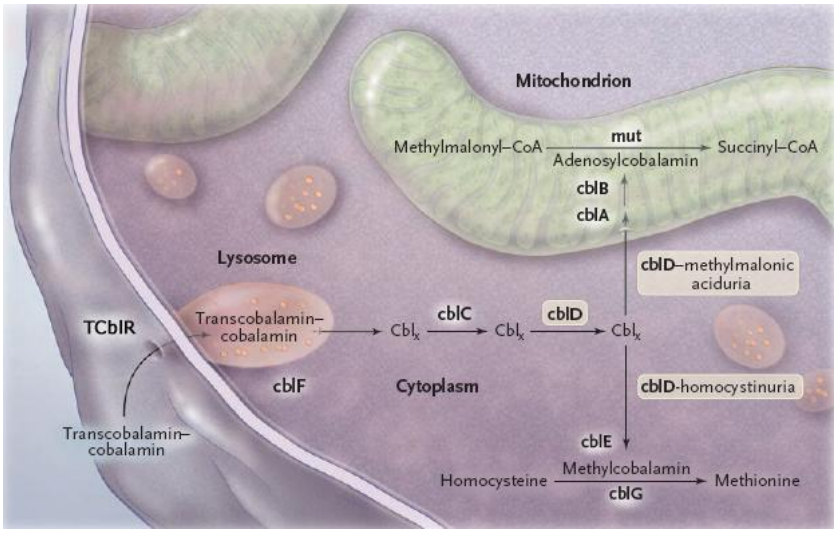

Figure 16. Intracellular cobalamin metabolism and its defects.

Cobalamin bound to transcobalamin II enters the cell by means of lysosome-mediated endocytosis via transcobalamin-cobalamin receptor (TCblR) and is released by proteolysis. Export from the lysosome into the cytoplasm is defective in patients with the cblF defect. The steps in the cytosol after lysosomal release are still unclear but are defined by the complementation groups cblC and cblD. In addition, the exact form of cobalamin at this stage is unclear (as indicated by "Cblx"). In the cytoplasm, cobalamin is reductively methylated by methionine synthase reductase (cblE) to methylcobalamin, the cofactor for methionine synthase (cblG). In mitochondria, cobalamin is converted to adenosylcobalamin, the cofactor for methylmalonyl-coenzyme A (CoA) mutase (MUT), by cobalamin adenosyltransferase (cblB). The exact role of the protein associated with the cblA complementation group is unclear. The cblD protein (MMADHC) constitutes a branch point between the cytosolic and mitochondrial pathways, leading to three phenotypes: cblD-methylmalonic aciduria, cblD-homocystinuria and the combination of these.

Figure modified from (75).

2.2.4.3 Methylmalonic acid and methylmalonic aciduria

These parts are discussed in detail in section 2.1.5.

2.2.4.4 Homocysteine and hyperhomocysteinemia

Homocysteine, a sulphur-containing amino acid, is not obtained with the diet; it is produced in the cells as an intermediate in one-carbon metabolism. Methionine is converted to S-adenosylmethionine (SAM) by S-adenosylmethionine synthase in an ATP-dependent reaction. SAM acts as a methyl-donor for many methylation reactions within the cells forming S-adenosylhomocysteine (SAH). S-adenosylhomocysteine hydrolase releases the adenosine forming homocysteine (Figure 17). As homocysteine is potentially toxic, two mechanisms help to keep intracellular levels of homocysteine low (<10 µmol/l); a cellular export mechanism and intracellular catabolism of homocysteine (235, 236). A variety of homocysteine species exist in human plasma such as protein (albumin)-bound, free circulating disulfide and sulfhydryl forms (237). Current laboratory methods detect the presence of all three forms and report this as total homocysteine concentration.

Intracellular homocysteine metabolism represents an intersection of two pathways: remethylation to methionine by methionine synthase, a reaction requiring folate and vitamin B_{12} (or betaine in an alternative reaction catalyzed by betaine-homocysteine methyltransferase, although this pathway is only present in the liver (238)) and transsulfuration to cystathione, which requires pyridoxal-5'-phosphate (vitamin B_6). The two pathways are coordinated by *S*-adenosylmethionine (SAM), which can act as an inhibitor of the methylenetetrahydrofolate reductase (MTHFR) and as an activator of cystathionine β-synthase (Figure 17).

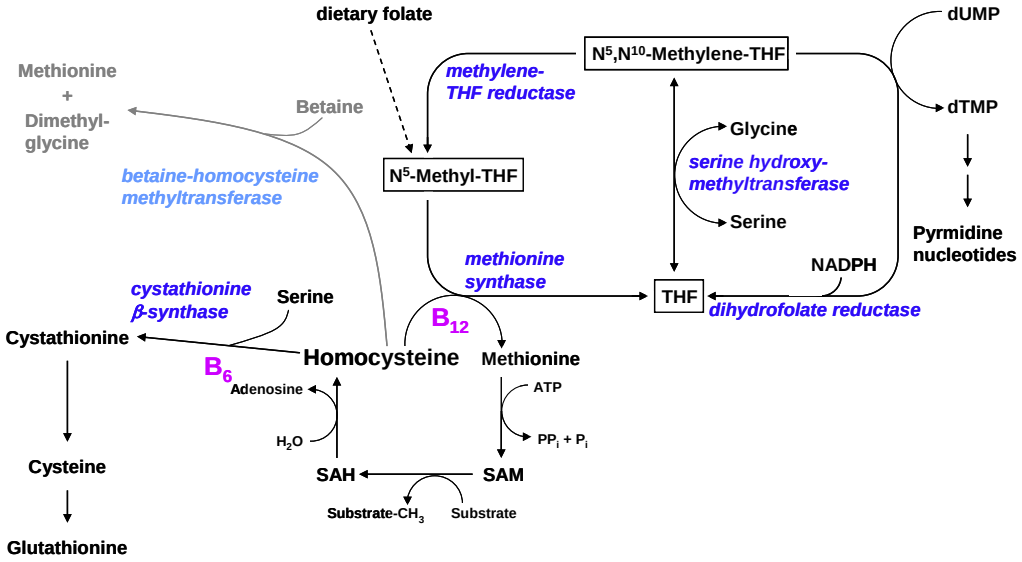

Figure 17: Homocysteine represents an intersection of two pathways: remethylation to methionine by methionine synthase, a reaction requiring folate and vitamin B_{12}; and transsulfuration to cystathionine requiring vitamin B_6. The reaction betaine dependent conversion of homocysteine to methionine and dimethyl-glycine carried out by betaine-homocysteine methyltransferase is only present in liver and kidney and represents a possible treatment option for hyperhomocysteinemia in these organs. SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine, THF: tetrahydrofolate.

Homocysteine metabolism is nutritionally regulated: while the amount of homocysteine degraded via the transsulfuration pathway decreases when the subjects are on a methionine restricted diet, excess dietary methionine leads to degradation of homocysteine via the transsulfuration pathway with the levels falling below baseline (10 µmol/l) (239).

This coordination seems to be achieved by two mechanisms, first SAM can act as an inhibitor of MTHFR as well as an activator of cystathionine β-synthase. Thereby, SAM suppresses the synthesis of N^5-Methyl-THF, an important substrate for remethylation, while at the same time promoting the initial reaction of transsulfuration. Secondly, the synthesis of SAM is regulated by two isoforms of S-adenosylmethionine synthases, one with a high affinity for methionine, the other with a low affinity for methionine. The two enzymes function at different methionine concentrations and have a different rate at which they can synthesize

SAM (240). Additionally, SAM seems to be regulated by glycine methyltransferase, an enzyme involved in the methyl-transfer from SAM to glycine forming methylglycine. This enzyme is abundant in the liver and is strongly inhibited by N^5-methyltetrahydrofolate. Thus, beside intracellular methionine, N^5-methyltetrahydrofolate also participates in the regulation of intracellular SAM concentrations (241). Part of the intracellular regulation of homocysteine metabolism is depicted in Figure 18.

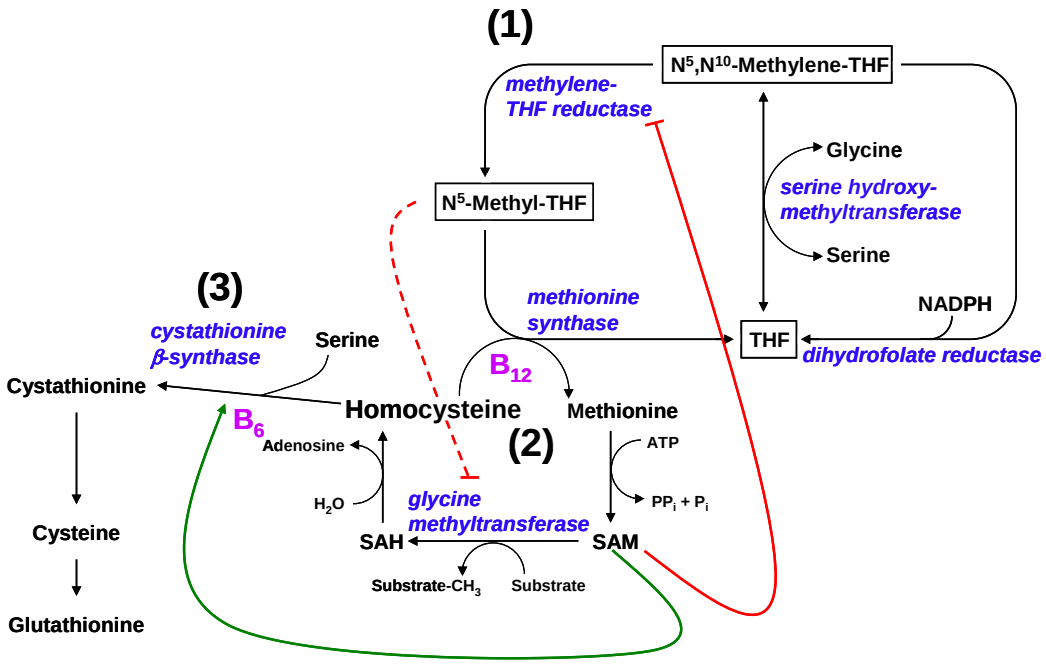

Figure 18: The regulation of intracellular homocysteine concentration: Homocysteine is a substrate for methionine synthase forming methionine in a vitamin B_{12}-dependent reaction. Methionine again is a precursor of SAM, an essential methyl-donor. High levels of SAM exert inhibitory effects on MTHFR and at the same time activate the B_6-dependent cystathionine β-synthase. SAM itself is converted to S-adenosylhomocysteine (SAH) by transfering its methyl group to various substrates. Reactions carried out by multiple methyltransferases. As SAH is a potent inhibitor of most methyltransferases, glycine methyltransferase is inhibited by high levels of N5-methyltetrahydrofolate, making sure that the ratio of SAM/SAH remains high to assure methylation.

Genetic defects in one of these steps or nutritional deficiency of one or more vitamins crucial for homocysteine metabolism can lead to hyperhomocysteinemia.

Hyperhomocysteinemia is caused by accumulation of homocysteine and is diagnosed by elevated levels of homocysteine in plasma and urine. The various forms of the disease can be categorized due to cause, prevalence and severity.

Defects in intracellular cobalamin metabolism leading to hyperhomocysteinemia include the cblF, cblC and cblD defect that lead to a combination of methylmalonic aciduria and homocystinuria and the cblE and cblG defects that lead to isolated homocystinuria. The clinical and biochemical phenotype of cblE and cblG patients is virtually identical. Affected patients suffer from severe developmental delay, ataxia, neonatal seizures and blindness.

They excrete high amounts of homocysteine while levels of methylmalonic acid are normal (72).

Severe hyperhomocysteinemia with levels up to 400 µmol/l is also caused by defects in cystathionine β-synthase (CBS) or MTHFR (242-244). In CBS deficiency the transsulfuration pathway is severely affected, thus the rate of methionine synthesis is increased leading to an increase in intracellular concentration of SAM. High levels of SAM cause feedback inhibition of MTHFR, thereby blocking both pathways involved in the degradation of homocysteine (245, 246). Clinical phenotypes of CBS deficiency also include ectopia lentis due to disruption of disulfide bonds in cysteine-rich fibrillin and marfanoid habitus due to abnormalities in collagen crosslinking (244, 247).

In MTHFR deficiency, homocysteine can be degraded solely by the transsulfuration pathway. However, low intracellular methionine levels lead to a decrease in intracellular SAM concentration lowering the efficiency of the transsulfuration pathway. As SAM is necessary for polyamine synthesis and for the methylation of numerous essential cell components such as phospholipids, methyl-accepting proteins, CpG islands in DNA, adrenergic, dopaminergic and serotoninergic molecules, SAM deficiency leads to a multitude of disorders. Furthermore SAM also feeds the transsulfuration pathway converting homocysteine to glutathione, the main cellular antioxidant required for detoxification of various cellular compounds and for the scavenging of free radicals (248).

Low intracellular SAM concentrations have been shown to lead to increased susceptibility to steatosis and oxidative liver injury, spontaneous development of steatohepatitis and hepatocellular carcinoma (249).

A genetic defect in any of the enzymes required for the synthesis of methyl-cobalamin, the cofactor of methionine synthase, leads to low intracellular SAM concentrations. Thus less homocysteine is being synthesized from SAM. The hyperhomocysteinemia resulting from impaired remethylation of homocysteine may not be as severe as that observed in MTHFR deficiency because transsulfuration is more active catabolizing homocysteine (250). The various defects leading to hyperhomocysteinemia are shown in Figure 18.

Mild hyperhomocysteinemia is caused by dietary folate or B_{12} deficiency or by moderate enzyme defects such as the common MTHFR polymorphism c.C677T (251).

A third phenotype is the post-methionine load hyperhomocysteinemia where total homocysteine increases only after a methionine load. Defects accounting for this phenomenon include dietary B_6 deficiency or a heterozygosity for a CBS defect (246).

Increasing age, impaired renal function, high levels of plasma creatinine, smoking, coffee consumption, alcoholism and certain drugs can also cause moderate increases in plasma homocysteine levels (252).

Hyperhomocysteinemia is associated with increased risk of developing a multitude of diseases including vascular diseases (253), arthrosclerosis (254), Alzheimer's disease (AD) (255) and end-stage renal disease (256).

As homocysteine metabolism in endothelial cells of human blood vessels relies exclusively on folate and vitamin B_{12}-dependent remethylation (257), the cardiovascular system is particularly sensitive to elevated levels of homocysteine. However, randomized clinical trials have shown that while vitamin supplementation substantially lowers the levels of circulating homocysteine, the outcome in the secondary prevention of cardiovascular disease or dementia is not improved (258).

Furthermore experimental evidence suggests that homocysteine can induce DNA damage in the CNS and may therefore play a role in ageing, cognitive decline and various forms of dementia including AD (259). DNA damage may be due to increased formation of reactive oxygen species (ROS). Intracellular homocysteine can reduce metal ions (e.g. Cu^{2+}) in a reaction that promotes the formation of reactive oxygen species (ROS). High concentrations of ROS can induce synaptic failure and may induce cellular changes associated with AD (260).

The underlying mechanism of homocysteine-induced end-stage renal disease is not fully understood but protein-bound homocysteine may reduce the glomerular filtration rate thereby further increasing circulating levels of homocysteine (261).

Treatment of genetic defects leading to hyperhomocysteinemia is limited. Depending on the nature of the defect, various forms of treatment can lower homocysteine levels and improve the outcome (262).

Treatment options are as follows (see Figure 18 for details):

- folates such as folic and folinic acid help maximize residual MTHFR activity
- the cofactor riboflavin can enhance the residual activity of MTHFR
- N^5-methyltetrahydrofolate can replace the missing product of MTHFR
- pyridoxine can activate cystathionine β-synthase
- OH- or Me-cobalamin can support MS or to help maximize residual MS activity
- betaine to support betaine-homocysteine methyltransferase
- methionine to replenish methionine, SAM and carnitine

2.2.4.5 Aims of the cobalamin project

The aim of this part of my thesis was to clone the wildtype and mutant cDNAs of various proteins involved in intracellular cobalamin metabolism. These constructs were transfected into immortalized fibroblasts from subjects with a defect in intracellular cobalamin metabolism as well as controls. By doing so, we investigated the effect of the various wildtype and mutant cDNAs on the synthesis of cobalamin cofactors and their implication on disease.

Chapter 1

Cryptic Exon Activation by Disruption of Exon Splice Enhancer

NOVEL MECHANISM CAUSING 3-METHYLCROTONYL-CoA CARBOXYLASE DEFICIENCY

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ABSTRACT

3-Methylcrotonyl-CoA carboxylase (MCC) deficiency is an autosomal recessive disorder of leucine catabolism. MCC is a heteromeric mitochondrial enzyme composed of biotin-containing α (MCCA) and smaller β (MCCB) subunits encoded by *MCCA* and *MCCB*, respectively. We report studies of the c.1054G→A mutation in exon 11 of *MCCB* detected in the homozygous state in a patient with MCC deficiency. Sequence analysis of *MCCB* cDNA revealed two overlapping transcripts, one containing the normal 73 bp of exon 11 including the missense mutation c.1054G→A (p.G352R), the other with exon 11 replaced by a 64 bp sequence from intron 10 (cryptic exon 10a) that maintains the reading frame and is flanked by acceptable splice consensus sites. In expression studies, we show that both transcripts lack detectable MCC activity. Western blot analysis showed slightly reduced levels of MCCB using the transcript containing the missense mutation, whereas no MCCB was detected with the transcript containing the cryptic exon 10a. Analysis of the region harboring the mutation revealed that the c.1054G→A mutation is located in an exon splice enhancer sequence. Using *MCCB* minigene constructs to transfect MCCB-deficient fibroblasts, we demonstrate that the reduction in utilization of exon 11 associated with the c.1054G→A mutation is due to alteration of this exon splice enhancer. Further, we show that optimization of the weak splice donor site of exon 11 corrects the splicing defect. To our knowledge, this is the first demonstration of a point mutation disrupting an exon splice enhancer that causes exon skipping along with utilization of a cryptic exon.

Accurate and efficient removal of introns from pre-mRNA is essential for gene expression. The information present in the consensus splice-site signals, the 5' splice-site, branch site, and 3' splice-site, is necessary but not always sufficient to define exon-intron boundaries (1, 2). On average, these signals appear to provide about half of the information required for exon and intron recognition in human transcripts (3). Sequences flanked by consensus splice-site sequences but not known to be retained in mature transcripts are common in introns. Some may be "cryptic exons" that for unknown reasons are not normally included in mature mRNAs; others may be "true" exons that have not been recognized because they are alternatively spliced in some developmental or tissue-specific fashion (1, 4, 5).

Additional *cis*-acting sequence elements that function as splicing enhancers and silencers exist in the genome (6). Exon splice enhancers (ESEs)[2] are short sequences within exons that augment exon recognition and the control of alternative splicing (1). ESEs bind

serine/arginine-rich (SR) proteins, a family of essential splicing proteins that activate splicing by defining exons and recruiting the splicing machinery to the adjacent intronic splice consensus sequence (6-8). ESEs appear to be common and are present in most, if not all, exons including constitutive exons (6, 9). Unlike transcriptional enhancers, ESEs function in a strongly position-dependent manner, enhancing splicing when present downstream of a 3' splice-site or upstream of a 5' splice-site. Other *cis*-acting sequences, often in introns, repress exon recognition (6, 10). In some instances, ESEs appear to compensate for “weak” 5' or 3' splice signals in introns. Strengthening of the splice consensus sites of an enhancer-dependent exon by site-directed mutagenesis may eliminate dependence on the enhancer (8, 10).

Single nucleotide substitutions in the coding regions of genes are the most commonly recognized type of mutation underlying inherited human diseases (11). The molecular pathophysiology of nonsense mutations results from a combination of premature termination of translation and nonsense-mediated mRNA decay (NMD) (12, 13). The NMD pathway is activated by nonsense mutations (or frame-shifting deletions or insertions) that occur 5' of the last exon-intron junction and minimizes the potential for problems caused by truncated proteins by reducing the abundance of the abnormal transcript (12, 13). The deleterious effects of missense mutations are usually attributed to their effects on protein stability, folding, or function (14), whereas synonymous mutations are often assumed to have no pathophysiologic consequences. Recently, our understanding of the potential pathological effects of single nucleotide substitutions has been expanded to include alterations that inactivate ESEs causing exon skipping (10, 15-17). The importance of considering the possibility of RNA-processing phenotypes in the analysis of the consequences of mutations is emphasized by the growing list of exonic variations that alter RNA-processing and thereby cause or modify disease (see the list of 34 missense and 26 “silent” point mutations causing exon skipping reviewed in Ref. 6).

3-Methylcrotonyl-CoA carboxylase (MCC, EC 6.4.1.4) is a heteromeric mitochondrial enzyme composed of biotin-containing α subunits and smaller β subunits, encoded by *MCCA* (*MCCC1*; Mendelian Inheritance in Man (MIM) 609010) and *MCCB (MCCC2*; MIM 609014), respectively (18). Mutations in these genes can cause isolated MCC deficiency (MIM 210200 and 210210), a disorder of leucine catabolism inherited as an autosomal recessive trait with a variable phenotype that ranges from neonatal-onset with severe neurological involvement to asymptomatic adults (11, 18, 19). Introduction of tandem mass spectrometry to newborn screening has resulted in a large increase in the number of inborn errors that can be detected, including several amino acidemias and organic acidurias. This technique has shown MCC deficiency to be one of the more frequently detected organic acidurias with an overall frequency of ~1:50'000 and a mainly mild phenotype (19, 20).

Here we report detailed studies of a missense mutation (c.1054G→A, p.G352R) in exon 11 of *MCCB* that disrupts an ESE and causes MCC deficiency by utilization of a cryptic exon instead of the normal *MCCB* exon 11. The patient in which this mutation was detected (MCC019) is the child of a consanguineous union and presented at 7 months with failure to thrive (19).

EXPERIMENTAL PROCEDURES

Cell Cultures and Carboxylase Assays - Skin fibroblasts were cultured in Dulbecco's (transfected cells) or Earl's minimal essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, and antibiotics. To be able to sequence unstable transcripts, NMD was inhibited by adding emetine (100 µg/ml) in the culture medium 10 h before harvesting the cells (21). Activities of MCC and propionyl-CoA carboxylase (PCC) were assayed in fibroblast homogenates by measuring the incorporation of [^{14}C]bicarbonate into acid non-volatile products with established methods (22).

Mutation Analysis by RT-PCR and Genomic PCR - We extracted RNA and genomic DNA from cultured fibroblasts using RNA and DNA isolation kits from Qiagen and performed RT-PCR using 2-5 µg of total cellular RNA with the cDNA cycle kit (Invitrogen) following the manufacturer's instructions. All PCR reactions (50 µl) contained primers (100 ng each), standard PCR buffer (Invitrogen), dNTPs (200 µM), and *Taq* polymerase (2.5 units; Invitrogen). The sequences of all primers are listed in Supplementary Table 1.

Construction of Wild-type and Mutant MCCB Expression Vectors - To introduce the c.1054G→A mutation and the transcript containing the cryptic exon (exon 10a) instead of exon 11, we amplified cDNA of patient MCC019 with primers DV4498 and DV4503 and subcloned the PCR products into the pTracer-*MCCB*-wild-type construct (18) by using the *Bst*EII and *Sfi*I sites.

MCCB Minigene Constructs - To examine splicing between exons 9 and 12 of the *MCCB* gene, we constructed an *MCCB* minigene by modifying a vector (pBK-RSV-*OAT*), which had previously been shown to function as an *in vivo* splicing template (12). First a multiple cloning site was introduced between the *Xba*I and the *Pst*I sites of the vector. Then an 8.9 kb fragment containing the genomic DNA sequence between the 3' end of exon 9 (DV5050) and the 5' end of exon 12 (DV5049) of *MCCB* was amplified from genomic DNA of patient MCC019. This sequence was now introduced into the multiple cloning site at the *Spe*I and *Sac*I sites (see Fig. 3*a*).

To optimize the donor splice-site of *MCCB* intron 11 (CAGgtataa), we used site-directed mutagenesis to change it into the consensus donor sequence (CAGgta*agt*) (see Fig. 3*a*). To do this, overlapping forward (DV5111) and reverse (DV5112) primers containing both the c.1054G→A mutation and an optimized intron 11 donor splice-site (c.1072+4 taa→agt) were

designed. Primer DV5111 was used with a reverse primer in intron 11 (DV5094), producing a 1952 bp genomic fragment containing a *Xma*I site. Primer DV5112 was used with a forward primer in intron 10 (DV5110), amplifying a 1381 bp genomic fragment containing a *Xca*I site. These two PCRs were combined and amplified by using the outer primers DV5110 and DV5094, producing a 3233 bp genomic fragment containing both the c.1054G→A mutation and an optimized donor splice-site of intron 11. This fragment was subcloned into pBK-*MCCB*-G352R using the *Xca*I and *Xma*I sites to obtain the pBK-*MCCB*-G352RΔss construct. Similarly, to introduce the wild-type sequence, we amplified a 3233 bp genomic fragment from control DNA and subcloned it into the pBK-*MCCB*-G352R construct. We verified that all the inserts and constructs had the indicated changes and stayed in-frame by sequencing all the exons and flanking intronic sequences.

Transfections - For expression studies, the constructs were transiently transfected into an immortalized MCCB-deficient reference cell line by electroporation as described (19). The reference cell line originates from skin fibroblasts of a patient homozygous for *MCCB* Q43X, shows no detectable MCC activity, and does not express detectable MCCB protein. We harvested the cells 48 h after transfection and assayed for MCC and PCC activities.

Protein Extraction and Western Blot - Cell lysates were prepared by harvesting confluent fibroblasts from a 75 cm^2 flask by trypsinization. The cells were washed with 5 ml of phosphate buffered saline and centrifuged for 5 min at 200 x *g*. The pellet was resuspended in 150 µl of protein extraction reagent (M-Per, Pierce) and homogenized in a mortar. The homogenate was centrifuged for 5 min at 14'000 x *g*. The supernatant was transferred into a new tube, and the protein concentration was measured using the A280 method (NanoDrop; Witec).

Proteins were separated by SDS-PAGE (50 µg per lane) and detected by immunoblotting. Anti-MCCB (Abnova) was used at a dilution of 1:1'000. Signals were detected using the ECL detection kit (Amersham Biosciences). 4 µg of protein was processed similarly as loading control and probed with a monoclonal anti-β-actin antibody (Sigma).

RESULTS

Patient MCC019 presented with severe isolated MCC deficiency shown by deficient MCC activity (2.2 % of median control value) together with normal PCC activity in homogenates of primary fibroblasts (Fig. 1). Virtually no MCCB protein was detected in fibroblasts by Western blotting (Fig. 1).

Sequence analysis of *MCCB* RT-PCR cDNA of this proband revealed two overlapping transcripts (19). One contained the normal 73 bp of exon 11 with a missense mutation c.1054G→A (p.G352R); in the other, exon 11 was replaced by a sequence of 64 bp from

intron 10 (*MCCB*-exon10a). This cryptic exon maintains the reading frame and is flanked by acceptable splice consensus sites (ctttagAAA.....ATGgtaagt; average score of 86 (23)).

Amplification and sequencing of exon 11 from genomic DNA showed that the patient was homozygous for c.1054G→A (Fig. 2*a*). However, we considered the possibility that the patient had a deletion of one *MCCB* allele being hemizygous for *MCCB*-p.G352R but rejected this possibility because the patient is the product of a consanguineous union and because Southern blot analysis of genomic DNA after digestion with several restriction enzymes showed normal amounts of *MCCB* and no fragments of abnormal size (not shown). These results indicated that the patient indeed carries the missense mutation on both alleles.

To determine the functional consequences of the mutation, we subcloned both transcripts into the pTracer vector and expressed them in an immortalized MCCB-deficient reference cell line (Fig. 1). Transfection of the *MCCB* wild-type construct restored MCC activity from less than 1 % of the median control value to 16 %. Constructs with *MCCB*-G352R and *MCCB*-exon10a showed no rescue of activity, confirming the functional significance of these transcripts.

Western blot of lysates of cells transfected with the *MCCB*-G352R construct revealed slightly reduced levels of MCCB protein as compared with those transfected with the wild-type construct. No protein was detectable after transfection with the *MCCB*-exon10a construct (Fig. 1). This finding, together with Western blot data of untransfected patient fibroblasts, indicates that both variants lead to protein that is less stable than the wild-type protein, and in the case of *MCCB*-exon10a, is rapidly degraded, causing absence of MCC activity.

To visualize and assess the relative abundance of the two transcripts, we used primers in exon 10 (DV5020) and 12 (DV5019) to amplify either a 114 bp fragment (containing exon 11) or a 105 bp fragment (lacking exon 11 but containing the cryptic exon 10a) from cDNA and separated the products on a 12 % acrylamide gel (Fig. 2*b*). From patient RNA, the 114 and 105 bp fragments were produced in the ratio of 3 to 2, respectively, whereas only the 114 bp fragment was produced from control RNA. Both fragments with similar relative levels were also obtained using RNA extracted from the lymphoblasts of the patient, indicating that this result is not specific for fibroblasts (not shown).

The effect of NMD on these steady-state levels of *MCCB* transcripts was investigated by repeating RT-PCR amplification in emetine-treated cells. The most abundant *MCCB* transcript in RNA from emetine-treated patient cells was one lacking both exon 11 and exon 10a (Fig. 2*c*). This 41 bp transcript (*MCCB*-Δ10a, 11) results in a frame-shift followed by a stop codon. It was detected only after amplification of the RNA of the patient after emetine treatment, indicating that it is rapidly degraded by NMD under normal circumstances.

The normal exon 11 motif TATGGA was identified as a putative SR protein, SRp55-responsive ESE, by two independent web-based ESE prediction programs, ESEfinder (24) and RESCUE-ESE (25), and has been shown to be capable of functioning as an ESE (26). This motif is mutated to TAT**A**GA in our patient. We therefore hypothesized that the reduction in utilization of exon 11 associated with the c.1054G→A mutation (Fig. 2*b*) is due to disruption of this ESE.

To confirm this hypothesis, we first investigated whether we can reproduce the skipping of exon 11 by expressing minigene constructs in an MCCB-deficient reference cell line (Fig. 3*a*). After transfection, we used primers complementary to *OAT*/exon9 (DV5114) and *OAT*/exon12 (DV5115) to amplify by RT-PCR a 292 bp fragment containing part of *MCCB* exon 9, exon 10, and exon 11 and part of exon 12. Sequencing of the products showed that the wild-type product had the expected size of 292 bp. In contrast, most of the product of G352R was 219 bp in length and lacked exon 11, a phenotype detected in the cells of the patient only after inhibition of NMD by emetine treatment. No fragment with the cryptic exon 10a in place of exon 11 was obtained (Fig. 3*b*).

ESEs have been associated with exons that have weak flanking splice-sites (1, 8), and the donor splice-site of intron 11 is indeed weak (a score of 68 as compared with 75-98 for most normal exons (23)). To determine whether the presence of a stronger donor site would reduce dependence on the ESE, we expressed a minigene construct containing the mutated ESE motif in combination with an optimized donor splice-site (G352RΔss, Fig. 3*a*). Optimization of the donor splice-site indeed corrected the splicing defect and led to amplification of a normal sized fragment (Fig. 3*b*). These results confirm our hypothesis that *MCCB* c.1054G→A partially disrupts an ESE and that a functional ESE is no longer required when the splice donor site matches the consensus sequence.

DISCUSSION

About half of the disease-causing nucleotide substitutions have been estimated to lead to aberrant splicing (6, 16, 27). Typically, the abnormal splicing results from inactivation of a splice-site or creation of a new splice-site or interference with regulatory *cis*-elements, such as splicing enhancers or silencers. Our study of the c.1054G→A (p.G352R) mutation, detected in the homozygous state in the *MCCB* gene of patient MCC019, illustrates a mechanism by which a missense mutation partially disrupts an ESE causing utilization of a cryptic exon (exon 10a) in place of exon 11. The c.1054G→A mutation is located in the centre of a putative ESE motif responsive to the human SR protein SRp55 (24, 25) and affects a highly conserved amino acid. Previous work has shown that ESEs can compensate for weak 5' or 3' splice signals in exons and that strengthening of the splice consensus sites of an enhancer-dependent exon generally eliminates enhancer dependence (10).

The inclusion of exon 10a in place of exon 11 does not change the reading frame and results in the replacement of the normal 24 amino acid sequence encoded by exon 11 by a novel sequence of 21 amino acid residues. Our expression studies clearly show that *MCCB*-exon10a produces no detectable MCCB, whereas some albeit slightly reduced levels of MCCB are produced by *MCCB*-G352R, suggesting that the *MCCB*-exon10a and to a lesser extent the *MCCB*-G352R lead to an unstable protein product. This is supported by less MCCB protein in patient fibroblasts as compared with control fibroblasts (Fig. 1). The discrepancy between the amount of MCCB protein seen in the fibroblasts transfected with wild-type MCCB and the level of MCC activity could probably be explained by the fact that transfection efficiency was quite low (9 %, data not shown), whereas at the same time, the few transfected fibroblasts produced massive amounts of MCCB protein due to the cytomegalovirus promoter present in the construct. The active MCC complex is a $(\alpha\beta)_6$ dodecamer, and overproduction of one of the subunits could lead to incomplete and therefore inactive or only partly active complexes.

The glycine at position 352 is a highly conserved amino acid, and the possibility that this change is a polymorphism has previously been ruled out (19). In addition, the region of MCCB encoded by exon 11 is thought to be part of the 3-methylcrotonyl-CoA binding site that gives the enzyme its substrate specificity (18). It is highly likely that replacement of 24 amino acids in this region and a missense mutation within this exon lead to failure of 3-methylcrotonyl-CoA binding with deleterious functional consequences.

The most common consequence of point mutations that affect splicing is exon skipping (16, 28). This is especially true for point mutations disrupting ESEs (6, 15, 16, 27, 29). In our patient, we were only able to observe exon skipping after suppression of NMD and in our minigene expression system. The explanation for the predominance of a transcript resulting from exon skipping rather than one with inclusion of exon 10a (as in untreated fibroblast RNA) in our minigene system remains unclear. Preliminary attempts to correct exon-skipping events by repairing the sequence of damaged exonic enhancers have been proven to be efficient *in vitro* and *in vivo* (30-32) and hold promise for future therapeutic use.

Cryptic exons have been shown to be activated by intronic mutations that either create or strengthen splice-sites, create a new branch site, or are located within a cryptic exon (5, 16, 33-36). To our knowledge, this is the first demonstration of an exonic point mutation disrupting an ESE that leads to skipping of the corresponding exon and at the same time activates utilization of a cryptic exon from the adjacent intron. Mutations causing inclusion of cryptic exons may be more prevalent than the current literature suggests. Potential cryptic exons are frequent in introns and in some genes greatly outnumber genuine exons but are normally not included in the mature mRNA (4). Detection of these events in disease may be

overlooked because introns are often excluded from mutation analysis and because it is often impossible or impractical to utilize cDNA for mutation analysis.

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[2] The abbreviations used are: ESE, exon splice enhancer; MCC, 3-methylcrotonyl-coenzyme A carboxylase; MCCA, methylcrotonyl-coenzyme A carboxylase α subunit; MCCB, methylcrotonyl-coenzyme A carboxylase β subunit; PCC, propionyl-coenzyme A carboxylase; NMD, nonsense-mediated mRNA decay; RT-PCR, reverse transcriptase-PCR; SR, serine/arginine-rich.

No potential conflict of interest relevant to this article was reported.

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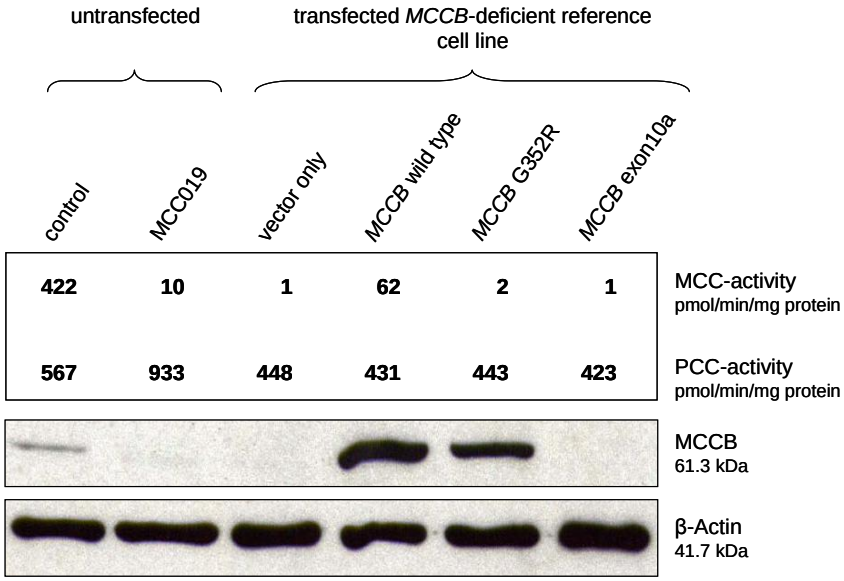

Figure 1: Expression of pTracer constructs with *MCCB* wild-type cDNA and both types of mutant cDNA of patient MCC019 (G352R and exon10a) in an immortalized MCCB-deficient reference fibroblast cell line.

Untransfected control and patient MCC019 fibroblasts were also analyzed. Transfections were performed by electroporation, and cells were harvested 48 h later for the assay of MCC and PCC activities as well as Western blot analysis of MCCB using β-actin as a control. Transfection with the empty vector (*vector only*) was used as a negative control. Values are the mean of parallel determinations from a representative experiment. For further details, see "Experimental Procedures. " Median control values and range (in brackets) for MCC and PCC activities in 30 different immortalized fibroblast cell lines are: MCC, 399 pmol/min/mg protein (220-683); and PCC, 660 (309-840).

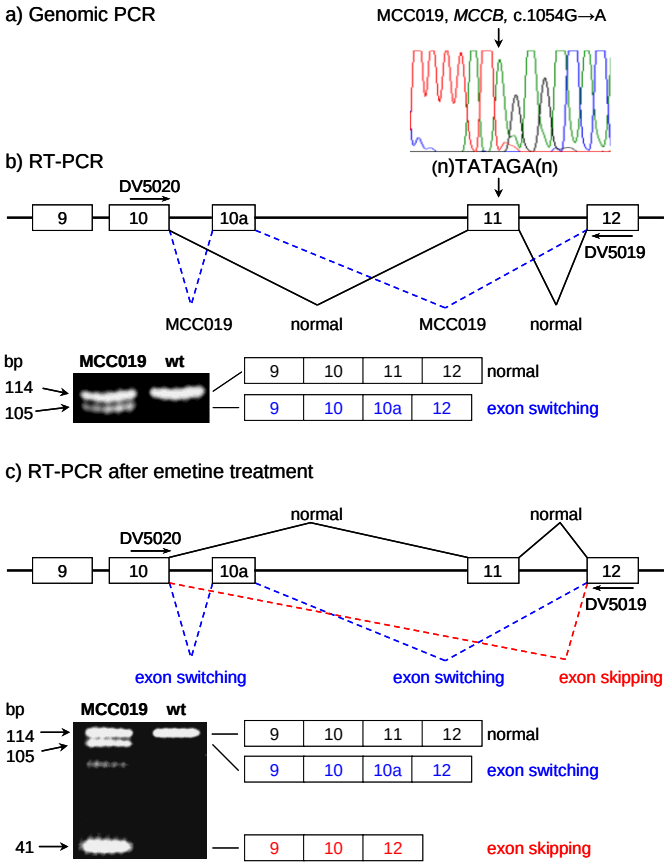

Figure 2: Schematic diagram of the splicing defect found in MCC019.

Boxes represent exons, and *dark horizontal lines* represent introns. *a*, genomic PCR of *MCCB* exon 11 in MCC019 showing the homozygous mutation. *b*, schematic diagram and RT-PCR products of the splicing variants. *wt*, wild-type. *c*, RT-PCR products after treating the cells with emetine (100 µg/ml) before harvesting the RNA. Emetine is used to inhibit nonsense-mediated mRNA decay (21).

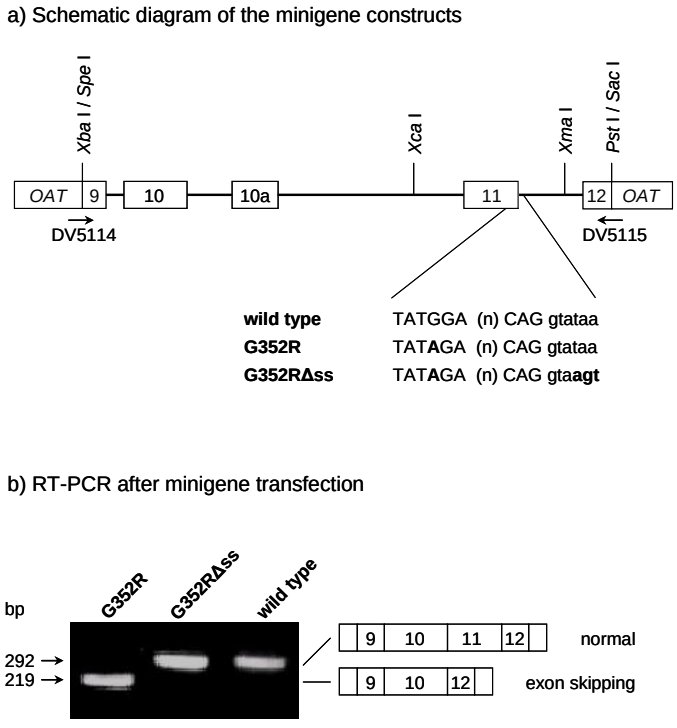

Figure 3: *In vivo* splicing analysis.

Boxes represent exons, and *dark horizontal lines* represent introns. The relevant portion of exon 11 and its 3'-flanking splice-site is shown for three minigene constructs: wild-type, c.1054G→A (G352R), and c.1054G→A with the optimized donor splice-site (G352RΔss).

Intronic sequences are shown in *lowercase*, and mutated nucleotides are shown in *bold*. *a*, schematic diagram of the three minigene constructs and the primers used for RT-PCR analysis. *b*, RT-PCR products after transfecting MCCB-deficient reference fibroblasts with the indicated minigene constructs and the corresponding exon arrangements.

Supplementary table 1: Sequences of all primers used in the study.

primer	sequence (5' - 3')
DV4498	cccagtttgcaggttaccag
DV4503	taggagcccccaatgatgag
DV5019	acccaaatattcgagcaaatcc
DV5020	ggagctttgatgtccgagag
DV5049	cttgccttttttgcagattcagaaaagagagctccgttgtttcc
DV5050	gggctttggatgatcatcatgcccttcacttaactagtgaaggttgtgagg
DV5094	ccaggctggaatgcaatgg
DV5110	atagttgttacgtgttgattgc
DV5111	gcagattcactgagttcaaagccttttatagagacacattagttacaggtaagtaggtgaagaattgaaaatacg
DV5112	gattttcaaagcagcagaaaatgttcgtattttcaattcttcacctacttacctgtaactaatgtgtctct
DV5114	attgaccttaacatctagtaagg
DV5115	cactggagagagagctccg

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Chapter 2

Gene Identification for the cblD Defect of Vitamin B_{12} Metabolism

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ABSTRACT

BACKGROUND

Vitamin B_{12} (cobalamin) is an essential cofactor in several metabolic pathways. Intracellular conversion of cobalamin to its two coenzymes, adenosylcobalamin in mitochondria and methylcobalamin in the cytoplasm, is necessary for the homeostasis of methylmalonic acid and homocysteine. Nine defects of intracellular cobalamin metabolism have been defined by means of somatic complementation analysis. One of these defects, the cblD defect, can cause isolated methylmalonic aciduria, isolated homocystinuria, or both. Affected persons present with multisystem clinical abnormalities, including developmental, haematologic, neurological, and metabolic findings. The gene responsible for the cblD defect has not been identified.

METHODS

We studied seven patients with the cblD defect, and skin fibroblasts from each were investigated in cell culture. Microcell-mediated chromosome transfer and refined genetic mapping were used to localize the responsible gene. This gene was transfected into cblD fibroblasts to test for the rescue of adenosylcobalamin and methylcobalamin synthesis.

RESULTS

The cblD gene was localized to human chromosome 2q23.2, and a candidate gene, designated *MMADHC* (methylmalonic aciduria, cblD type, and homocystinuria), was identified in this region. Transfection of wild-type *MMADHC* rescued the cellular phenotype, and the functional importance of mutant alleles was shown by means of transfection with mutant constructs. The predicted MMADHC protein has sequence homology with a bacterial ATP-binding cassette transporter and contains a putative cobalamin binding motif and a putative mitochondrial leader sequence.

CONCLUSIONS

Mutations in a gene we designated *MMADHC* are responsible for the cblD defect in vitamin B_{12} metabolism. Various mutations are associated with each of the three biochemical phenotypes of the disorder.

Vitamin B_{12} (cobalamin) is essential for normal development and survival in humans and must be obtained from animal products or supplements. Inside the cell, it is converted to two active cofactors, adenosylcobalamin and methylcobalamin (Fig. 1) (1). Adenosylcobalamin is the coenzyme for mitochondrial methylmalonyl-coenzyme A mutase, which converts L-methylmalonyl-coenzyme A to succinyl-coenzyme A and is involved in catabolism of odd-chain fatty acids and some amino acids. Methylcobalamin is the coenzyme for cytosolic methionine synthase, which converts homocysteine to methionine and is essential for normal one-carbon metabolism, which is in turn involved in vital cellular processes such as methylation and DNA synthesis (1). Disturbances of cobalamin-cofactor synthesis due to acquired or inherited alterations result in elevated levels of homocysteine and methylmalonic acid, which are associated with multisystem clinical abnormalities similar to those seen in patients with severe nutritional vitamin B_{12} deficiency, including lethargy, hypotonia, developmental delay, seizures, and megaloblastic anemia.

Inborn errors of cobalamin-cofactor synthesis represent a heterogeneous and important group of rare disorders. Intracellular cobalamin metabolism involves multiple steps between the lysosomal release of cobalamin and the synthesis of adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol. To date, nine distinct defects of this pathway have been defined in vitro with the use of somatic complementation analysis. The complementation groups thus identified have been designated cblA, cblB, cblC, cblD, cblE, cblF, cblG, cblH, and mut (Fig. 1) (2, 3) The responsible genes are known except for cblD, cblF, and cblH, although the function of some of the associated proteins is not clear. The cblC and cblF disorders cause combined homocystinuria and methylmalonic aciduria; cblA, cblB, cblH, and mut cause isolated methylmalonic aciduria; and cblE and cblG cause isolated homocystinuria. The cblD defect (Online Mendelian Inheritance in Man number, 277410) (2) is puzzling in that some patients have combined methylmalonic aciduria and homocystinuria (called “cblD original” by Suormala et al. (2) but herein called “cblD-combined”), some have only isolated homocystinuria (called “cblD variant 1” (2) or, herein, “cblD-homocystinuria”), and others have only methylmalonic aciduria (“cblD variant 2” (2) or, herein, “cblD-methylmalonic aciduria”). Here, we describe our identification of the cblD gene, confirmation of its identity by using biochemical and molecular studies, and demonstration of functionally significant mutations in patients with the cblD defect.

METHODS

PATIENTS AND CELL LINES

The study was performed between 2004 and 2007. Seven unrelated patients with the cblD defect were studied. Written informed consent was obtained from the parents of each patient,

and patients who could give assent did so. The study was approved by the local ethics committee.

Cultures of skin fibroblasts were obtained for diagnostic purposes, and referring physicians approved the use of the samples for our investigation of the origin of the disease. We immortalized fibroblasts using the plasmid pRNS1 (4) and electroporation (Gene Pulser II, BioRad). Immortalization did not markedly affect cellular function (data not shown).

The functional integrity of the methylmalonic acid pathway was evaluated by measuring the degree of incorporation of [^{14}C]propionate into macromolecules and formation of adenosylcobalamin from [^{57}Co]cyanocobalamin, as described previously (2). The integrity of methionine synthase in the homocysteine pathway was defined as the formation of methionine from [^{14}C]formate and synthesis of methylcobalamin from [^{57}Co]cyanocobalamin (2).

Somatic complementation analysis (2) confirmed that all fibroblast cell lines from patients belonged to the cblD group (including one that had been used to define cblD (5)). A fibroblast cell line from one of the seven patients had previously been assigned to a new complement group (cblH) by Watkins et al. (6), but we show here that it in fact belongs to the cblD complement group (Fig. 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org).

MICROCELL-MEDIATED CHROMOSOME TRANSFER

Mouse-human monochromosomal hybrid cell lines (donor cells), each carrying a single human chromosome tagged with a hygromycin resistance gene (7), were used to serially transfect one of the immortalized cblD-homocystinuria cell lines (recipient cells) with the use of microcell-mediated chromosome transfer, as described previously (8) (also see Table 1 in the Supplementary Appendix). Cells containing each transfected human chromosome were selected on the basis of growth in medium containing hygromycin. Colonies were subcultured and assayed for methylcobalamin synthesis. Those that showed such synthesis, and thus the rescue of cellular function, were defined as positive colonies; those that did not were defined as negative colonies.

GENE MAPPING

Positive colonies were tested for contamination by mouse DNA with the use of the mouse-specific microsatellite markers BAM5 and RSINE1 SINE/B4 (9, 10). Positive colonies that were free of mouse DNA contamination and negative colonies then were used in the fine mapping of the donor chromosome, by means of microsatellite markers, to define the chromosomal segment containing the putative gene corresponding to the cblD defect. The presence of these microsatellite markers was tested with the use of the polymerase-chain-

reaction (PCR) assay, involving fluorescently labeled primers. Products were visualized in an automated sequencer (ABI Prism 3100, Applied Biosystems) and analyzed with Genotyper software (version 2.5, Applied Biosystems). The chromosomal region defined by the microsatellite markers was examined for candidate genes, and a suitable candidate was selected on the basis of sequence characteristics.

DNA SEQUENCING AND MUTATION ANALYSIS

Total RNA was extracted from cultured fibroblasts with the use of the RNeasy Kit (Qiagen), and full-length complementary DNA (cDNA) for the candidate gene was amplified by means of a reverse-transcriptase PCR (RT-PCR) assay involving specific primers and was sequenced according to the ABI BigDye method (Applied Biosystems). To confirm mutations identified in the RT-PCR products, the corresponding exons were amplified through the PCR assay from genomic DNA, with the use of flanking intronic primers, and were sequenced.

EXPRESSION OF CANDIDATE GENE cDNA IN FIBROBLASTS

Constructs containing wild-type and mutant cDNA sequences for the candidate gene were prepared in pTracer-CMV2 or pcDNA3.2/V5 expression vectors (Invitrogen), as described previously (11). Constructs were transfected into immortalized fibroblasts by means of electroporation. Transfection efficiency was 5 to 22 %, as determined by estimating the proportion of cells co-expressing the green fluorescent protein from the pTracer construct. Rescue of cellular function was tested by measuring methionine and methylcobalamin synthesis or adenosylcobalamin synthesis.

STATISTICAL Analysis

We tested the statistical significance of the data on rescue of function by using the unpaired t-test (two-tailed), with Welch's correction for unequal variances, and GraphPad Prism software (version 4). P values less than 0.05 were considered to indicate statistical significance.

RESULTS

CLINICAL DATA

The clinical features of the seven patients are shown in Table 1. Two patients had the isolated homocystinuria phenotype, and two had the isolated methylmalonic aciduria phenotype. These four patients have been described previously (Table 1). The three other patients, two of whom are newly described in this study, had the combined phenotype (methylmalonic aciduria and homocystinuria).

GENE LOCALIZATION

To identify the chromosome containing the cblD gene, we transfected immortalized cblD-homocystinuria cells (from Patient 2) with individual human chromosomes contained in mouse-human single-chromosome hybrid cell lines using microcell-mediated chromosome transfer. We then tested the resulting cell lines for rescue of function by measuring methylcobalamin synthesis. Transfection with chromosome 2 yielded 48 colonies that showed correction of methylcobalamin synthesis (positive colonies) and 24 colonies that did not (negative colonies) (Table 1 in the Supplementary Appendix). The positive colonies were screened for mouse-specific sequences, and four were found to be free of contamination with mouse DNA.

A panel of 38 microsatellite markers spanning chromosome 2 was used to perform fine mapping of the chromosome fragments from the 4 positive colonies and 22 of the negative colonies (see Table 2 in the Supplementary Appendix). This fine-mapping procedure identified a 10.2 Mb DNA segment between markers D2S150 and D2S2324 that was present in DNA from positive colonies but absent in all the negative colonies (Fig. 2 in the Supplementary Appendix). This mapped the cblD gene to the chromosome region 2q22.1-2q23.3 (Fig. 2A), which contains 28 genes (according to the deCODE genetic map; for details, see the accession numbers and URLs in Note 1 in the Supplementary Appendix). The *C2orf25* gene was selected as a candidate because of its homology (14) to a putative mitochondrial ATPase component of a bacterial ATP-binding cassette (ABC) transporter (YP_218380) and because cobalamin transport in bacteria is facilitated by an ABC transport system (15).

MUTATION ANALYSIS

The full-length cDNA for the candidate gene *C2orf25* was amplified by means of RT-PCR and specific primers (Table 3 in the Supplementary Appendix). The *C2orf25* gene was then sequenced from cDNA from each of the seven patients with the cblD defect, and mutations for each patient were identified. These mutations were further confirmed with the use of PCR amplification of the appropriate exons from genomic DNA, involving flanking intronic primers (Table 3 in the Supplementary Appendix). We identified nine mutations, with two mutant alleles in each patient (Table 1 and Fig. 2B).

Five of the mutations we identified are predicted to lead to a premature stop codon, resulting in a truncated protein: two nonsense mutations (160C→T, 748C→T), two deletions (57_64del, 696+1_4del), and one duplication (419dupA). The 696+1_4del splice-site mutation was shown by means of RT-PCR to cause the skipping of exon 7. This mutation was present in the homozygous state in Patient 7 and was found in the heterozygous state in both parents and in an at-risk fetus in this family (data not shown).

One in-frame duplication (307_324dup) was identified. This mutation is predicted to add six amino acids to the protein product.

Three mutations causing a single amino acid change were found (545C→A, 746A→G, and 776T→C), each occurring in a region of the gene that is highly conserved among species (Fig. 3). These three missense mutations occurred in patients of European origin and did not occur in 100 control chromosomes from subjects with the same ethnic backgrounds, substantially reducing the likelihood that these are common polymorphisms. Parental DNA, used to rule out a deletion in the homozygous patients, was available from only one family.

TISSUE EXPRESSION AND PROTEIN CHARACTERIZATION

We searched databases of known expressed sequences of the human genome to ascertain the tissue expression of the *C2orf25* gene. This gene appears to be expressed at high levels in most tissues (Note 2 in the Supplementary Appendix). To detect possible multiple transcripts, we amplified *C2orf25* from fibroblast messenger RNA (mRNA), by using RT-PCR and various primers. We found only a single cDNA product, with a coding region of 891 bp, encoding a putative polypeptide of 296 amino acids and a predicted molecular weight of 32.8 kDa (Fig. 3). Residues 1 through 12 constitute a possible mitochondrial leader sequence, which suggests targeting of the expressed protein to mitochondria (16). Residues 81 though 86 match the vitamin B_{12} binding motif, Asp-Xaa-His-Xaa-Xaa-Gly, constituting a putative binding site for cobalamin (17).

EXPRESSION OF HUMAN *C2orf25* cDNA IN FIBROBLASTS

To prove that the *C2orf25* gene is responsible for the cblD phenotype, we tested the ability of wild-type and mutant constructs to rescue cellular function (Fig. 4). The wild-type construct rescued methionine synthesis and methylcobalamin synthesis, which were both restored to 52 to 100 % of the control values in both cblD-homocystinuria and cblD-combined fibroblasts with the use of the pTracer vector (Fig. 4A and 4B). Adenosylcobalamin synthesis was not reproducibly corrected with the use of the pTracer wild-type construct. To test for rescue of adenosylcobalamin synthesis, we repeated the experiment with the pcDNA3.2/V5 vector containing the wild-type construct and showed restoration of adenosylcobalamin synthesis to 68 % of the control value in a cblD-combined cell line (Fig. 4C).

Constructs containing the missense alleles associated with isolated homocystinuria (545C→A, 746A→G, and 776T→C) did not restore methionine or methylcobalamin synthesis in either cblD-homocystinuria or cblD-combined cells, confirming that these mutant alleles cause the homocystinuria phenotype (Fig. 4A and 4B). Both constructs containing the mutations found in isolated methylmalonic aciduria (57_64del and 160C→T) rescued the synthesis of methylcobalamin (Fig. 4B).

Together, these findings suggest that mutations in the *C2orf25* gene are responsible for the cblD defect. We have therefore designated the gene *MMADHC,* or methylmalonic aciduria, cblD type, and homocystinuria (to indicate either the isolated phenotype or the combined phenotype).

DISCUSSION

We show evidence that mutations of the gene *C2orf25*, which we have designated *MMADHC*, cause the cblD defect in vitamin B_{12} metabolism. Our evidence includes the identification of nonconservative mutations in *MMADHC* in each of seven patients with cblD defects and the demonstration that expression vectors containing the wild-type *MMADHC* gene rescue cblD function in fibroblast cell lines. The predicted MMADHC protein contains the putative cobalamin binding motif and a putative mitochondrial leader sequence.

A complete analysis of the cblD defect requires an explanation of how mutations in the candidate gene lead to three distinct biochemical phenotypes. Although our data as described do not fully satisfy this requirement, our results suggest that the nature and location of the mutations are correlated with the biochemical phenotype, as illustrated in Figure 2B. Mutations found in the patients with cblD-methylmalonic aciduria are located toward the N-terminal part of the protein and consist of a nonsense mutation, a duplication, and a frame-shift deletion. Mutations found in the patients with cblD-homocystinuria are located toward the C-terminal part of the protein and consist of three missense mutations. Mutations found in the patients with the cblD-combined phenotype are located toward the C-terminal part of the protein and consist of a nonsense mutation, a splice-site deletion, and a frame-shift duplication.

Explaining these associations fully will require further studies. However, we offer the following speculations. The two patients with the cblD-methylmalonic aciduria defect carry mutations that are predicted to lead to truncated proteins of 19 amino acids in length (57_64del) or 53 amino acids in length (160C→T). We propose that the Met62 codon acts as a second start codon and leads to the reinitiation of translation (18), resulting in the formation of a shorter functional cblD protein product that lacks the putative mitochondrial leader sequence but allows for normal methylcobalamin synthesis. This would explain the isolated methylmalonic aciduria without homocystinuria in Patients 3 and 4 and is in accordance with the rescue of methionine and methylcobalamin synthesis in cblD-homocystinuria and cblD-combined cell lines transfected with the mutant alleles 57_64del and 160C→T. In support of this “reinitiation of translation” hypothesis, a strong Kozak consensus sequence (19) occurs at cDNA positions 184−3 and 184+4 (A at position-3 and G at position +4) in the *MMADHC* gene.

Both patients with isolated homocystinuria carry missense mutations (545C→A, 746A→G, and 776T→C) that are sufficient to cause deficient synthesis of methylcobalamin. However, we speculate that these mutations allow for the formation of a modified protein with an intact functional domain for the synthesis of adenosylcobalamin.

The patients with combined homocystinuria and methylmalonic aciduria carry mutations (419dupA, 696+1_4del, and 748C→T) that are predicted to lead to premature stop codons. We presume that these mutations would result either in a defective protein lacking both functional domains or in a low, steady-state abundance of *MMADHC* mRNA by the mechanism of nonsense-mediated mRNA decay.

These interpretations imply that at least two functional domains are present in the cblD protein and that the nature and location of the mutations correlate with the biochemical phenotype. However, this hypothesis remains speculative until proved in more patients. An alternative hypothesis is that even under normal conditions, two proteins are produced. However, we find no evidence of two different transcripts, and a single transcript of *MMADHC* rescues both biochemical phenotypes. This gene can be added to the list of genes known to be associated with multiple phenotypes involving various subcellular compartments, such as the amnionless gene (20) and the multiple sulfatase gene (21). To our knowledge, *MMADHC* is unique in that the three distinct biochemical phenotypes involve both the cytosolic and mitochondrial pathways.

The protein sequence of MMADHC is highly conserved among various mammalian species (Fig. 3). *MMADHC* is not a member of any previously identified gene family but was initially selected because of homology to a putative bacterial ABC transporter.

Although the segment of 91 amino acids between residues 78 and 168 shares 28 % identity and 46 % similarity with a putative ATPase component of an ABC transporter from *Salmonella enterica* (YP_218380), the cblD protein lacks critical motifs of ABC transporters such as Walker A, Walker B, and an ABC signature (22). This makes it unlikely that the cblD protein is a classic ABC transporter. ABC transporters are a diverse family of proteins with multiple functions; MMADHC may be a new type of ABC transporter or may be involved in a complex that facilitates transport in a fashion similar to that of ABC transporters.

It is not known how cobalamin enters the mitochondria in humans. Both active diffusion and passive diffusion have been suggested (23, 24). In some bacteria, cobalamin transport is facilitated by two processes. First, it is transported through the outer membrane by the BtuB transporter (15), which is mediated by TonB (25), a protein that couples energy from the proton motive force to the transport of cobalamin. Second, it is transported through the inner membrane by the BtuFCD ABC transport system (22). The C-terminal region of the human cblC protein (MMACHC) folds in a manner similar to that of TonB from *Escherichia coli* (26), and the human cblD protein MMADHC, the existence of which we have predicted, shares

some sequence identity with an ABC transporter from *S. enterica.* It is tempting to speculate that the *MMACHC* and *MMADHC* genes may have evolved to carry out functional roles that are similar to those of their bacterial counterparts, by forming structurally similar proteins.

In conclusion, we studied seven patients with the cblD defect in vitamin B_{12} metabolism. We found that mutations in a gene we designated *MMADHC* are responsible for this defect, and we demonstrated that various mutations are associated with each of the three biochemical phenotypes of the disorder.

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The pRNS1 immortalized control fibroblast cell line was a gift from Prof. Viktor Kozich and Dr. Petra Zavadakova (Prague, Czech Republic).

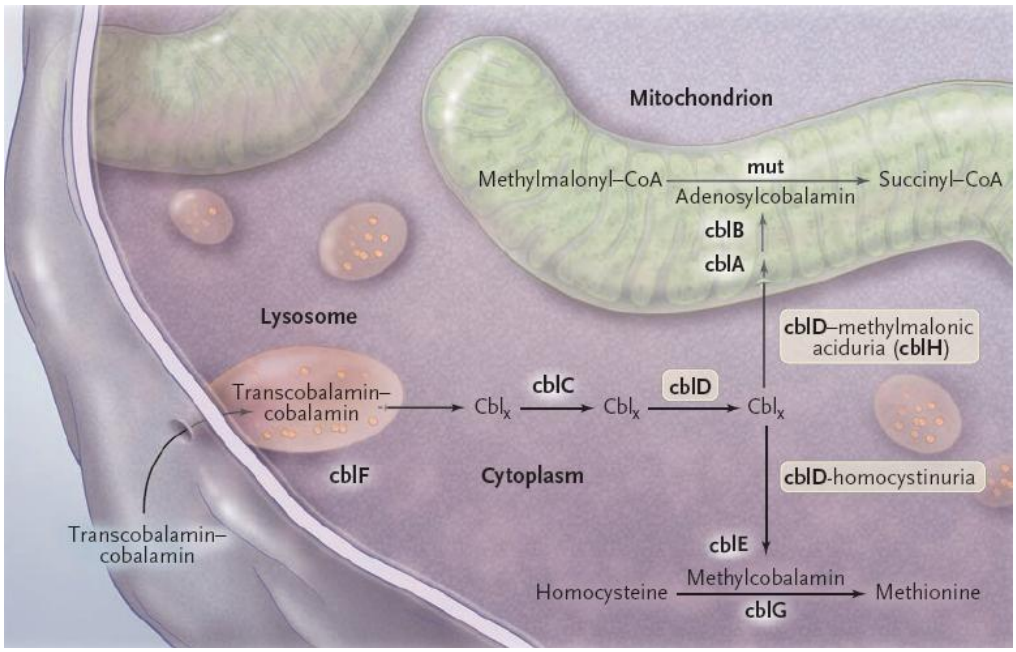


Figure 1: Intracellular Cobalamin Metabolism and Its Defects.

To date, nine complementation-group defects of the cobalamin pathway have been described. Cobalamin bound to transcobalamin enters the cell by means of lysosome-mediated endocytosis and is released through proteolysis. Export from the lysosome into the cytoplasm is defective in patients with the cblF defect. The steps in the cytosol after lysosomal release are still unclear but are defined by the complementation groups cblC and cblD. In addition, the exact form of cobalamin at this stage is unclear (as indicated by "Cbl_x"). In the cytoplasm, cobalamin is reductively methylated by methionine synthase reductase (cblE) to methylcobalamin, the cofactor for methionine synthase (cblG). After its transport into the mitochondrion, cobalamin is converted to adenosylcobalamin, the cofactor for methylmalonyl-coenzyme A (CoA) mutase (mut), by cobalamin adenosyltransferase (cblB). The exact role of the protein associated with the cblA complementation group is unclear. Our studies show that the cblD protein constitutes a branch point between the cytosolic and mitochondrial pathways, controlled by the cblD-methylmalonic aciduria variant and the cblD-homocystinuria variant, respectively. We also show that the cblH complementation group is identical to the cblD-methylmalonic aciduria group.

Patient No. and Mutant Alleles†	Predicted Amino Acid Mutations	Ethnic Group‡	Sex	Parental Consanguinity	Biochemical Phenotype	Age at Diagnosis	Clinical Findings Leading to Diagnosis	Study
1								
776T→C (both alleles)	Leu259→Pro	Irish	M	Yes	Homocystinuria	6 Yr	Global developmental delay, spastic ataxia, gait problems, delayed visual evoked potentials, increased mean corpuscular volume	Suormala et al.[2] (Case 1)
2								
545C→A, 746A→G	Thr182→Asn, Tyr249→Cys	Italian	M	No	Homocystinuria	3 Mo	Hypotonia, nystagmus, dystonia, seizures, megaloblastic anemia	Suormala et al.[2] (Case 2)
3								
57_64delCTCTTTAG (both alleles)	Cys19fsX20	Indian	M	No	Methylmalonic aciduria	Unknown§	Preterm birth at 32 wk of gestation, grade II respiratory distress syndrome, necrotizing enterocolitis, neonatal convulsions	Suormala et al.[2] (Case 3)
4								
160C→T, 307_324dup	Arg54X, Leu103_Ser108dup	Haitian	M	No	Methylmalonic aciduria	11 Mo	Severe ketotic coma, dehydration, hyperammonemia, leukopenia, thrombocytopenia	Cooper et al.[3]
5								
748C→T (both alleles)	Arg250X	Spanish-American	M	Yes	Methylmalonic aciduria and homocystinuria	14 Yr	Acute psychotic episode, marfanoid appearance, nystagmus, increased mean corpuscular volume, mild mental retardation	Goodman et al.[12] (Case 1) and Carmel and Goodman[13] (Patient J.R.)
6								
419dupA (both alleles)	Tyr140X	Scandinavian	F	Unknown¶	Methylmalonic aciduria and homocystinuria	3 Mo	Developmental delay, seizures, megaloblastic anemia	This study
7								
696+1_4delGTGA (both alleles)	Phe204_Ala232del	Italian	M	Yes	Methylmalonic aciduria and homocystinuria	22 Days	Poor feeding, encephalopathy, seizures, increased mean corpuscular volume	This study

Table 1: Mutations in Complementary DNA in Seven Patients with the cblD Defect.*

* Fibroblast cultures from Patients 4 and 5 were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital (where the patients were identified as WG1437 [cblH] and WG1220 [the original patient with the cblD defect], respectively). Fibroblast cultures from Patients 6 and 7 were supplied by Dr. B. Woldseth (Oslo, Norway) and Dr. D. Ketteridge (Adelaide, Australia), respectively.

† Mutation numbering is based on the complementary DNA sequence (NM_015702) with +1 corresponding to the A of the ATG translation-initiation codon. The 696+1_4delGTGA mutation results in exon 7 being skipped.

‡ Ethnic group was reported by the parents of the patients.

§ Elevated excretion of methylmalonic acid was detected and treated shortly after birth, but enzymatic diagnosis was performed only at the age of 8 months.

¶ The parents of this patient were from the same village.

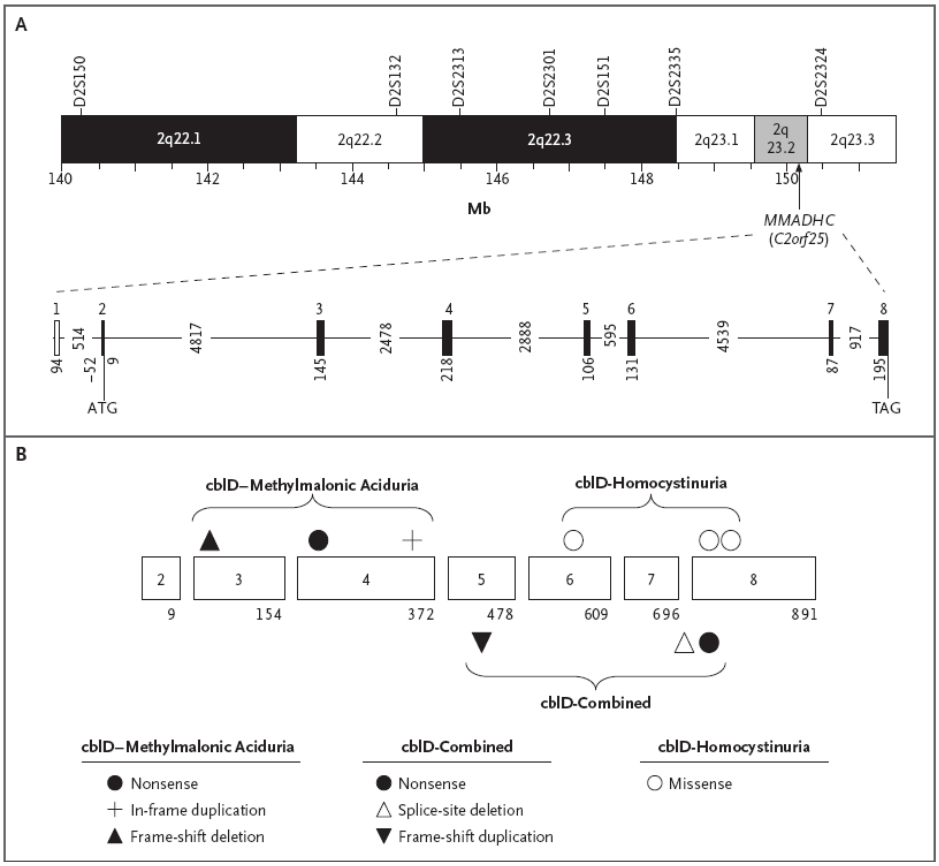


Figure 2: Location, Structural Organization, and Mutations of *MMADHC* (Methylmalonic Aciduria, cblD Type, and Homocystinuria).

Panel A shows the fragment of chromosome 2, between markers D2S150 and D2S2324, that was identified by means of microcell-mediated chromosome transfer and microsatellite mapping. This fragment is 10.2 Mb in length. The position of the markers is indicated according to the National Centre for Biotechnology Information's human reference sequence, build 36.1. *MMADHC* (currently named *C2orf25*; locus NM_015702) is located in chromosome 2 (genomic sequence NT_005403) and maps to chromosome region 2q23.2. The gene consists of eight exons. Three in-frame stop codons precede the first initiation codon (ATG) in exon 2. Exon 1 and the 5′ end of exon 2 are not translated. Exons 2 through 8 encode an 891 bp message, from the initiation codon to the TAG termination codon, predicted to encode a polypeptide of 296 amino acids with a predicted molecular mass of 32.8 kDa. The coding regions are indicated as black vertical bars with exon numbers above and the size in base pairs below; intron sizes, in base pairs, are indicated between the exons. Panel B represents composite complementary DNA (cDNA) for *MMADHC* and shows the location of mutations, grouped according to the cblD phenotype. Exons are represented by the numbered boxes, and the numbers below the boxes indicate the position within the cDNA sequence of the last nucleotide of each exon.

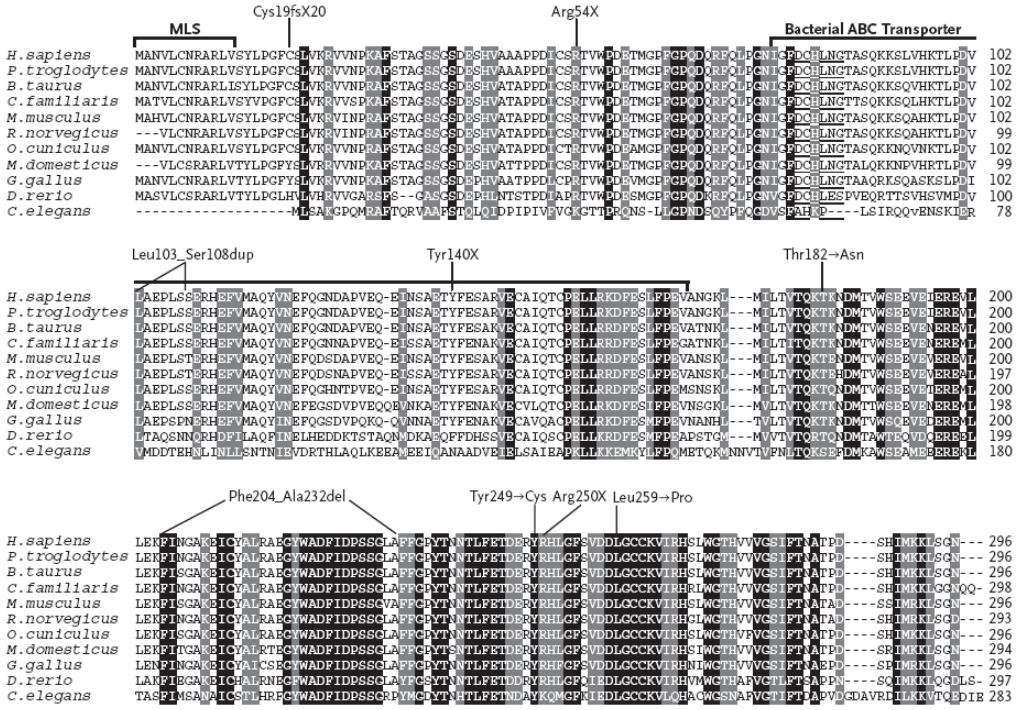


Figure 3: Evolutionary Conservation of MMADHC (Methylmalonic Aciduria, cblD Type, and Homocystinuria).

Amino acid sequence alignment for MMADHC and its orthologues was performed with the use of ClustalW software. Residues that are identical in all species listed are shown in black; residues with conservative substitutions are shown in grey. The portion of the sequence with genetic identity to a bacterial hypothetical mitochondrial ATP-binding cassette (ABC) transporter is indicated. The amino acids constituting the putative cobalamin binding motif are underlined (residues 81-86). Dashes represent amino acids that are absent in the protein of the particular species. The value along the right side of the sequences indicates the number of the last amino acid shown in the row. MLS denotes the putative mitochondrial leader sequence, as predicted by means of the Mitoprot II program. Sequences are shown for the following species (top to bottom in each group): *Homo sapiens, Pan troglodytes, Bos taurus, Canis familiaris, Mus musculus musculus, Rattus norvegicus, Oryctolagus cuniculus, Mus musculus domesticus, Gallus gallus, Danio rerio, and Caenorhabditis elegans.*

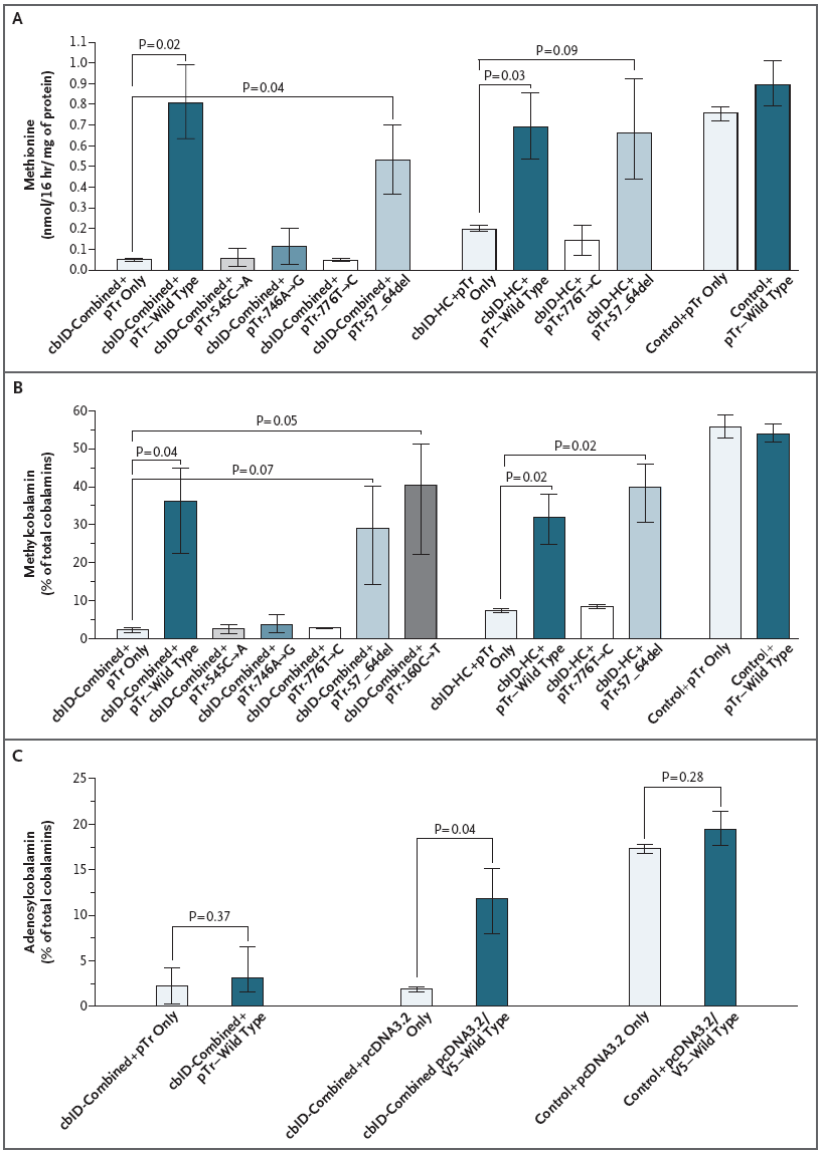
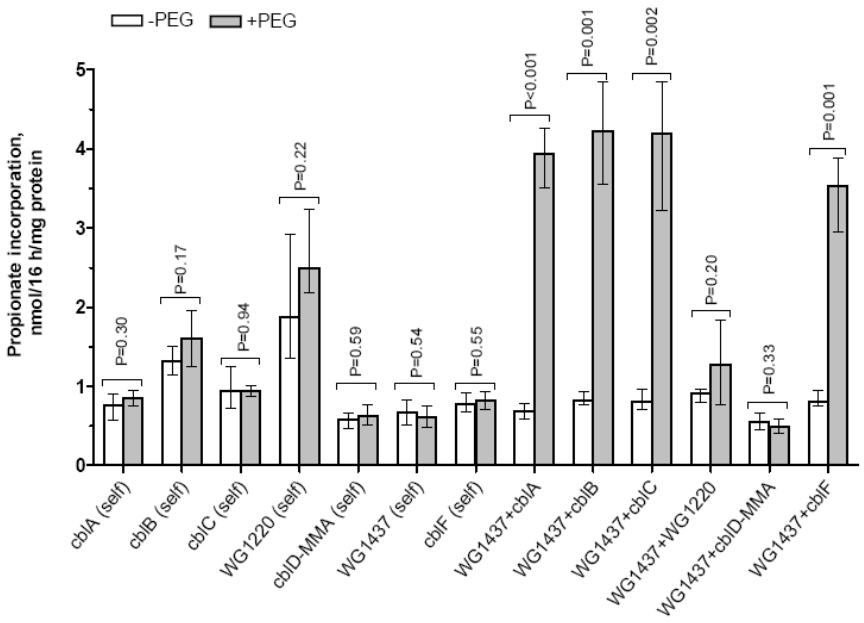


Figure 4: Expression of Wild-Type and Mutant Alleles of Human *MMADHC* (Methylmalonic Aciduria, cblD Type, and Homocystinuria) in Immortalized cblD-Combined, cblD-Homocystinuria, and Control Fibroblasts.

Cells from Patient 7 with the cblD-combined phenotype (homozygous for 696+1_4delGTGA), Patient 2 with the cblD-homocystinuria phenotype (cblD-HC) (compound heterozygous for missense mutations 545C→A and 746A→G), and one control were immortalized and used for transfection experiments. Transient transfection was performed by means of electroporation with pTracer (pTr) vectors containing an *MMADHC* allele: the wild-type allele, one of the three missense alleles (545C→A, 746A→G, or 776T→C) associated with cblD-HC, or the frame-shift deletion 57_64delCTCTTTAG or the nonsense mutation 160C→T associated with cblD-methylmalonic aciduria. Background activity was measured in cells transfected with empty pTracer vector (pTr only). Panel A shows the formation of methionine from [^{14}C]formate, and Panels B and C show the synthesis of methylcobalamin, and adenosylcobalamin, respectively, from [^{57}Co]cyanocobalamin. Transfection of the cblD-combined cell line and the control cell line was also performed with the pcDNA3.2/V5 vector containing wild-type *MMADHC*. Background activity was measured in cells transfected with empty pcDNA3 vector (pcDNA3.2 only). The data are means, and the bars indicate the range of results of single determinations from each experiment. For Panels A and B, the values are from three replicate experiments, and P values are shown only for comparisons indicating rescue of function. For Panel C, values for the pTracer vector are from seven replicate experiments, and those for the pcDNA3.2 vector are from three replicate experiments.



Supplementary Figure : Somatic complementation analysis in cell line WG1437 (No. 4 in this paper) from a patient with isolated methylmalonic aciduria, previously classified as cblH.

Complementation analysis was performed as described in reference 2 using primary fibroblasts and reference cell lines belonging to the known complementation groups associated with methylmalonic aciduria, i.e. cblA, cblB, cblC, cblF, the original cblD (cell line WG1220 from a patient with combined homocystinuria and methylmalonic aciduria) and cblD-MMA (cell line No. 3 from a patient with isolated methylmalonic aciduria). These cell lines were mixed pair-wise with the cell line WG1347 and fused with polyethyleneglycol (PEG, shaded columns +PEG) and 4 days later incorporation of [^{14}C]propionate into acid precipitable material was determined. Parallel cultures of mixed unfused cells were assayed as background controls (unshaded columns -PEG). Self fusions were used as negative controls. Columns represent mean values and the vertical lines are ranges from 4 replicate experiments, each with a single determination. P-values are derived from the unpaired t-test (two-tailed) with Welch's correction for unequal variances. WG1437 cells clearly complement the reference cell lines, cblA, cblB, cblC and cblF but not the WG1220 or cblD-MMA cells. These findings were confirmed by Dr. David Watkins (Montreal) and establish that WG1437 belongs to the cblD complementation group.

Supplementary Table 1: Cobalamin coenzyme synthesis after transfection of immortalized cblD-homocystinuria (cblD-HC) fibroblasts with human chromosome 2 by microcell mediated chromosome transfer (MMCT).

	% of total cobalamins*			
Cell line	OH-Cbl	MeCbl	AdoCbl	CN-Cbl
cblD-HC, transfected, 48 positive colonies#	25 ± 5 (17- 39)	44 ± 9 (20 –61)	21 ± 7 (10-36)	10 ± 5 (3 – 24)
cblD-HC, transfected, 24 negative colonies#	36 ± 7 (23 -47)	6 ± 2 (3 -10)	44 ± 12 (23 -70)	14 ± 7 (3 - 26)
Control, not transfected§	19 ± 4 (12-26)	44 ± 10 (26-63)	21 ± 6 (12-34)	16 ± 3 (11-20)
cblD-HC, not transfected§	32 ± 6 (21-42)	5 ± 2 (1-8)	54 ± 9 (41-70)	9 ± 4 (3-15)
Donor cells with human chromosome 2§	26, 26	19, 37	18, 20	38, 17

* Percentage of total cobalamins extracted from cells cultured for 2 days in medium containing [^{57}Co]cyanocobalamin; OH-Cbl, hydroxocobalamin; MeCbl, methylcobalamin, AdoCbl, adenosylcobalamin; CN-cbl, cyanocobalamin.

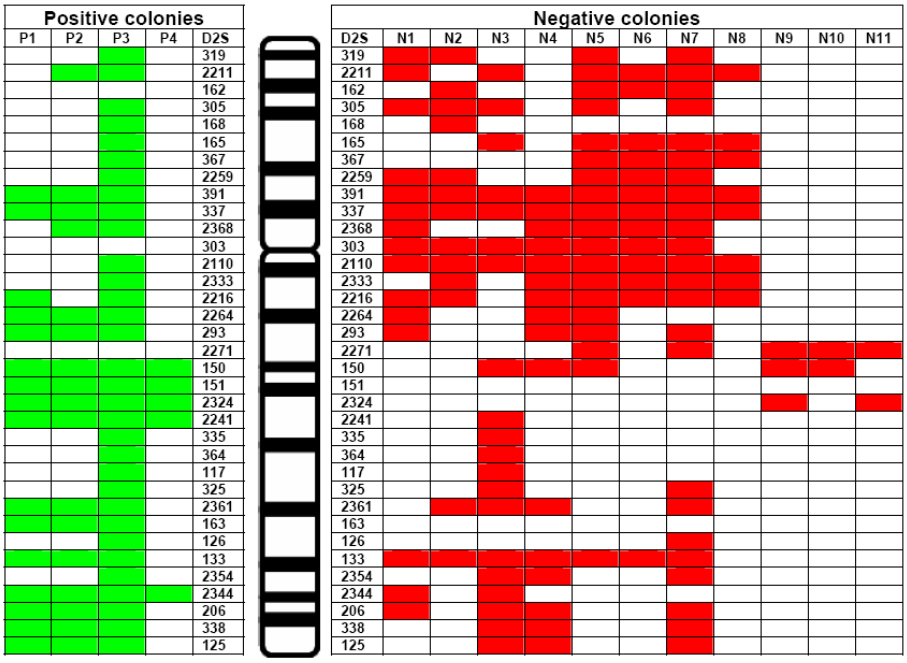
#Colonies were obtained after transfer of human chromosome 2 from the mouse/human chromosome 2 hybrid cell line (donor cells) into immortalized fibroblasts of cblD-HC (patient No. 2) by MMCT. Microcells were produced from the donor cells by inducing micronucleation with colcemid treatment for 48-66 hours (0.05 mg/l; KaryoMAX, Gibco) and by incubation and centrifugation in the presence of cytochalasin B (10 mg/l; Sigma). They were purified by serial filtration through 8 µm and 5 µm polycarbonate membranes (Magna® PCTE, Osmonics). Attachment of microcells to donor cells was facilitated by lectin (0.1 g/l PHA-P, Sigma) and cells were fused by treatment with polyethyleneglycol 1450 (Sigma). Cells containing the transfected human chromosome were selected by growth in medium containing hygromycin (0.1 mg/l, Calbiochem), subcultured and assayed for cobalamin coenzyme synthesis. Values are shown for positive colonies which rescued the function and negative colonies which did not, and are expressed as the mean ± SD with range in brackets. Methylcobalamin synthesis in negative colonies is not different from that in not transfected cblD-HC cells ($P=0.16$), whereas in positive colonies it is significantly increased ($P<0.001$) and virtually identical to that in the control ($P=0.91$).

§ For not transfected immortalized control and cblD-HC fibroblast cell lines, values are the mean ± SD with range in brackets from 10 and 19 replicate experiments, respectively, with single determinations. For donor cells values are from individual experiments with single determinations.

Supplementary Table 2: Markers used for mapping of chromosome 2.

Marker	Physical Position	DeCODE (cM)	Genethon (cM)
D2S319	4965347	7.6	6
D2S2211	7388831	ND	14
D2S162	8810895	22.73	21.3
D2S168	11396073	ND	28.6
D2S305	19340079	ND	40.7
D2S165	28515130	ND	50.7
D2S367	34352799	57.89	58.3
D2S2259	42850145	67.06	67.4
D2S391	46323154	72.33	73.8
D2S337	61581582	83.98	84.1
D2S2368	67062334	89.24	89.2
D2S303	70374279	ND	92.1
D2S2110	72983920	97.2	95.1
D2S2333	85341684	108.81	107.7
D2S2216	88190785	111.48	115.3
D2S2264	101878139	116.13	118.1
D2S293	106732296	119.77	122.4
D2S2271	127816132	140.48	137.4
D2S150	140279920	151.96	154.4
D2S151	147620286	157.5	156.4
D2S2324	150461036	160.82	158.6
D2S2241	154338317	163.27	161.9
D2S306	160387947	167.91	170.3
D2S335	172274835	177.98	182.5
D2S364	182860040	187.67	192.9
D2S117	195444305	194.63	201.4
D2S325	208096289	204.88	210.9
D2S2321	208099506	204.88	212.7
D2S2361	210526737	212.25	217.2
D2S163	220617879	221.36	225.6
D2S126	221842473	223.35	228.8
D2S133	224460495	227.17	231.7
D2S2354	227174810	229.16	235
D2S362	229563256	233.85	238.3
D2S2344	233270199	239.32	245.7
D2S206	233533331	240.03	248.3
D2S338	237017412	248.37	258.7
D2S125	240888116	258.19	269.5

These markers were selected from Linkage Mapping Set v2.5 (Applied Biosystems) and from deCODE Genetics and Genethon genetic maps. ND: not determined.



Supplementary Figure 2: Fine mapping of chromosome 2 fragments.

The human polymorphic DNA markers included in the panel are depicted in the columns labeled D2S. Other columns show the results obtained with the various colonies and each line corresponds to a different marker. Results obtained with positive colonies are represented on the left side of the table and those obtained with negative colonies are on the right. Shaded areas indicate the presence of a marker in DNA from the particular colony. The only marker present in all positive colonies and absent in all negative colonies is D2S151 located between D2S150 and D2S2324. Complete mapping was performed for the 11 negative colonies shown here. Fine mapping was performed with selected markers in another 11 negative colonies. The marker, D2S151 was absent in all negative colonies.

Supplementary Note 1: accession codes and URLs

C2orf25: Unigene Hs.5324, NM_015702, NP_056517.

Putative ATPase component of ABC transporter with ATPase domain: YP_218380.1.

Orthologs of MMADHC used for alignment in Figure 2:

Pan troglodytes, NP_001004280.1; *Bos taurus*, NP_001029713; *Canis familiaris*, XP_533352; *Mus musculus*, NP_598600.1*; Rattus norvegicus*, NP_001004280.1; *Oryctolagus cuniculus*, Ensembl Translation ID ENSOCUP00000007023 ; *Monodelphis domestica*, Ensembl Translation ID ENSMODP00000004357 ; *Gallus gallus*, CAG31701.1; *Danio rerio*, NP_991157; *Caenorhabditis elegans*, NP_499801.

URLs

Mitoprot II software: http://ihg.gsf.de/ihg/mitoprot.html

Predotar software: http://urgi.versailles.inra.fr/predotar/french.html

TargetP software: http://www.cbs.dtu.dk/services/TargetP

Mitopred software: http://bioinformatics.albany.edu/~mitopred

MultiLOC software: http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc

PSORT II software: http://psort.nibb.ac.jp/form2.html

iPSORT software: http://hc.ims.u-tokyo.ac.jp/iPSORT

HSLPred software: http://www.imtech.res.in/raghava/hslpred

ESLPred software: http://www.imtech.res.in/raghava/eslpred

Primer 3.0 software: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

DeCODEgenetic map: http://www.decode.com

Supplementary Table 3: Primers used to amplify *MMADHC* exons and cDNA.

Exon	5'-3' Sense	5'-3' Antisense	PCR Product size (bp)
1	CTCCTATCCATCCACCCAAA	AAGACAGACGGACAGGATGG	371
2	ACCTTCGAAGAGTGGACGTT	ATACCACCCAATGCCATCAT	240
3	TTGAAAAGTACATAGGCTTTTACCC	CCAAAAGGAGATTTGTTTCAGG	648
4	AACGGACTTGCCTGTGATCT	GGACAATGCCAAGTTTTCCA	584
5	TTGAACTTCCAAATCTTATATTCATTG	GCAGCATGAGGTCAGATGTC	486
6	ACCCTTTTCAAAAATAACATACATTT	AAAGCTGCACTTTTACTCTCAAAAA	456
7	CAGTCTAATTTGCCATTGGTTTT	TTTCTTTGGGTAAAATTGTGAGA	354
8	AGGCGGTTTAAGTGTTGCAT	ATCCCAAATCACTTGCCAAT	668
cDNA	GTTCTGGGACAGCTGGAGAC	CAATGTGGATGTGTTCAACG	1188

Supplementary Note 2: tissue expression.

Based on expressed sequence tags (ESTs) examined in the dbEST database, UniGene (www.ncbi.nlm.nih.gov) and a comprehensive annotation of *C2orf25* in AceView (The AceView genes: www.ncbi.nlm.nih.gov/IEB/-Research/Acembly: Danielle and Jean Thierry-Mieg, Michel Potdevin, Mark Sienkiewicz. AceView: Identification and functional annotation of cDNA-supported genes in higher organisms), the *MMADHC* gene appears to be expressed at high levels in most tissues. 1'437 ESTs from multiple tissues were listed in the UniGene cluster Hs.5324 including brain, skin, bone marrow, testis, bladder, kidney, vascular tissue, esophagus, muscle, salivary gland, prostate, placenta, liver, lung, mammary gland, embryonic tissue, lymph, cochlea, ascites, uterus, blood, rectum, small intestine, whole brain, pharynx, bone, connective tissue, stomach, thymus, uncharacterized tissue, tonsil, heart, cervix, pancreas, colon, lymph node, umbilical cord, ovary, parathyroid, eye, pituitary gland, adipose tissue, spleen, mouth, trachea, adrenal gland and nerve.

According to AceView, 8 different splice products are produced encoding 8 different putative protein isoforms, three of which (transcript a, b and c) are complete cDNAs matching the genome and each supported by more than 100 EST clones. Transcript c is an mRNA of 1415 bp with an ORF of 891 bp encoding a putative polypeptide of 296 amino acids identical to 15 different clones (AF131802, CR626278, CR624911, CR612420, CR611592, CR596944, CR595331, CR594994, CR592888, BC000932, AF060224, BC010894, BC023995, BC022859, AF161510). In contrast, according to AceView, the putative transcripts a and b encode slightly larger proteins of 380 and 330 amino acids, which are each supported only by a single clone (CR596944 and CR595331, respectively). Both include transcript c but in addition have 84 amino acids at the N-terminus (transcript a) or 34 amino acids in the C-terminal part of the polypeptide (transcript b). The 3 transcripts appear to differ because an internal intron (intron 1) is not always spliced out (transcript a) or because an additional exon (after exon 6) is present (transcript b).

To experimentally verify this *in silico* data we performed RT-PCR amplification of fibroblast mRNA using primers in the 5'UTR and 3'UTR of the different putative transcripts. Only transcript c could be amplified. Further, we performed RT-PCR experiments using primers flanking the additional exon of the putative transcript b, but were not able to amplify this transcript. From these experiments we conclude that transcript c was the only authentic transcript in fibroblasts, a tissue in which this gene is expressed with measurable activity.

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Chapter 3

Translational Initiation at Multiple AUGs can explain the three Phenotypes found in the cblD Defect of Intracellular Cobalamin Metabolism

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ABSTRACT

BACKGROUND

The cblD defect of intracellular vitamin B_{12} metabolism leads to three distinct biochemical and clinical phenotypes. Depending on the location of the mutation in the *MMADHC* gene, the cell is still capable of forming methylcobalamin in the cytosol or adenosylcobalamin in mitochondria, the two cofactors required for methionine synthase and methylmalonyl-CoA mutase, respectively. Defects in *MMADHC* can thus lead to either isolated methylmalonic aciduria (cblD-MMA) or homocystinuria (cblD-HC), or, if both pathways are affected, to combined methlymalonic aciduria and homocystinuria (cblD-MMA/HC). This study focused on the elucidation of how mutations in *MMADHC* lead to three phenotypes. We provide evidence that reinitiation of translation at downstream AUGs explains the phenotypes found in cblD patients.

METHODS

We studied the effect of various constructs on rescue of adenosyl- and methylcobalamin formation. Expression vectors containing the wildtype *MMADHC* were modified by PCR to include an enhanced mitochondrial leader sequence or mutations changing possible downstream sites of reinitiation or mutations introducing stop codons. The constructs were transfected into cell lines derived from cblD patient's fibroblasts. *In vitro* transcription/translation was used to investigate reinitiation of translation.

RESULTS

In cblD-MMA/HC cells, improving mitochondrial targeting of MMADHC clearly increased the formation of adenosylcobalamin with a concomitant decrease in methylcobalamin formation. In cblD-MMA cells, this effect was dependent on the mutation found in the corresponding cell line and showed a negative correlation with endogenous *MMADHC* mRNA levels. Transcriptional initiation at Met116 is sufficient for rescue of methylcobalamin formation and the sequence between Met62 and Met116 seems important for rescue of adenosylcobalamin formation.

CONCLUSIONS

These new findings support our hypothesis that the nature and location of *MMADHC* mutations correlate with the phenotype and that a delicate balance exists between cytosolic MeCbl and mitochondrial AdoCbl synthesis, supporting the role of cblD protein as a branching point in intracellular cobalamin trafficking. Furthermore our data

indicate that the sequence after Met116 is sufficient for MeCbl synthesis whereas the sequence between Met62 and Met116 is required for AdoCbl synthesis.

In mammals vitamin B_{12} (cobalamin) is essential for normal development and survival and must be provided in the diet from animal products or from supplements. It is converted intracellularly to two active coenzyme forms, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). AdoCbl is the coenzyme for mitochondrial methylmalonyl-CoA mutase (MIM 609058) which converts L-methylmalonyl-CoA to succinyl-CoA and is involved in catabolism of odd-chain fatty acids and some amino acids. MeCbl is the coenzyme for cytosolic methionine synthase (MIM 156570) which converts homocysteine to methionine and is essential for normal one-carbon metabolism (1).
Increases of plasma homocysteine are associated with homocystinuria (HC) and with a number of disease states such as vascular disease, birth defects, cancer and neuro-psychiatric disorders linking disorders of cobalamin metabolism to common diseases (2-6).
Accumulation of L-methylmalonyl-CoA leads to methylmalonic aciduria (MMA) that has been associated with various disease states such as acute metabolic decompensation with encephalopathy that can lead to death, chronic failure to thrive, developmental retardation and renal failure (7,8).

To date, nine different complementation classes causing defects of intracellular cobalamin metabolism have been described (complementation groups cblA, cblB, cblC, cblD-MMA, cblD-HC, cblD-MMA/HC, cblE, cblF and cblG) (9-11). The cblC, cblD-MMA/HC and cblF disorders cause combined homocystinuria and methylmalonic aciduria (MMA/HC), the cblA, cblB and cblD-MMA isolated MMA and the cblD-HC, cblE, cblG isolated HC with variable clinical presentation.
The cblD defect is unique in that it is associated with three distinct biochemical phenotypes. Three individuals have been identified with deficient synthesis of both cobalamin coenzymes causing combined methylmalonic aciduria and homocystinuria (cblD-MMA/HC), two with deficiency of MeCbl synthesis and isolated homocystinuria (cblD-HC) and two with deficient synthesis of AdoCbl and isolated methylmalonic aciduria (cblD-MMA) (11). Clinical presentation is variable with onset ranging from infancy to adolescence. CblD-MMA/HC patients presented with development delay, seizures, hypotonia, lethargy and megaloblastic anemia, CblD-HC patients with development delay, ataxia and megaloblastic anemia and cblD-MMA patients had respiratory distress, cranial haemorrhage, seizures and an abnormal EEG (9,11). The gene responsible for the cblD phenotype was designated *MMADHC* (methylmalonic aciduria, cblD type, and homocystinuria).
While individuals suffering from cblD-MMA have at least one allele carrying a nonsense mutation in the N-terminal part of the protein, patients with cblD-HC carry missense

mutations located in the C-terminal part of the protein. The combined phenotype is associated with deleterious nonsense and missense mutations in the middle of the protein (9).

To exert its two functions, MMADHC most likely localizes to both, mitochondria and cytosol. A common mechanism to achieve dual localization besides alternative splicing is the use of organellar targeting sequences. It has been shown that the mitochondrial leader sequence (MLS) can shift the balance between mitochondrial and cytosolic dual localization (12). To direct proteins, both natural and synthetic, to mitochondria, the MLS of aldehyde dehydrogenase 2 (ALDH2), known for its efficient mitochondrial import, has been widely used in experimental settings (13).

Another possibility of dual localization is that ribosomes miss the first AUG (start) codon and start translation at another in-frame downstream initiation codon producing a shorter protein with the same C-terminus (14-16). This additional site of translational initiation can be another in-frame AUG (17,18) or even a codon other than AUG as it has been described in plants, yeast and mammalian cells (19-26).

In this study we show that reinitiation of translation at a downstream AUG can explain the phenotype found in cblD-MMA patients. We provide evidence that the sequence between Met62 and Met116 is important for the synthesis of mitochondrial AdoCbl and the sequence after Met116 is sufficient for cytosolic MeCbl synthesis. Further, we report five new cblD patients and show functional expression of four new mutations (c.60insAT, c.133dupG, c.683C>G and c.737A>G) which further confirm our proposed genotype-phenotype correlation.

METHODS

CELL CULTURE

Fibroblasts were obtained for diagnostic purposes from the patients with inform consent. The referring clinicians approved the use of the cells for our investigation of the origin of the disease. Fibroblasts were routinely grown in Earl's minimal essential medium (Amimed, Basel, Switzerland) supplemented with 10 % fetal bovine serum (Amimed) as described earlier (27). Fibroblasts were immortalized using the plasmid pRNS1 (28) and electroporation.

MOLECULAR GENETIC STUDIES

Genomic DNA and total RNA were extracted from cultured fibroblasts using the QIAamp DNA Mini Kit and RNeasy Kit (Qiagen, Hombrechtikon, Switzerland), respectively. For mutation analysis genomic DNA and cDNA synthesized from total RNA by reverse

transcription (RT-PCR) were PCR amplified using specific primers as described earlier (9). Sequencing was performed by the ABI BigDye method (Applied Biosystems, Rotkreuz, Switzerland).

PREPARATION OF CONSTRUCTS FOR TRANSFECTION

Constructs containing the MMADHC wildtype and mutant cDNA were prepared in pTracer-CMV2 expression vectors as described earlier (29). Shortly, cDNA was produced from total RNA by reverse transcription using the Titan One Tube RT-PCR Kit (Roche, Basel, Switzerland), cloned into pCR-Blunt-II-TOPO (Invitrogen, Basel, Switzerland), subcloned into pTracer-CMV2 (Invitrogen) at the *Eco*RI site, propagated in *E.coli* DH5α competent cells (Invitrogen). Plasmids for transfections were isolated using the QIAfilter Plasmid Maxi Kit (Qiagen).

By using oligonucleotides, the mitochondrial leader sequence of aldehyde dehydrogenase 2 (*MLRAAARFGPRLGRRLLSAAATQA* (30) was added to wildtype MMADHC cDNA at different positions. The same procedure was used to N-terminally truncate MMADHC. Mutations were inserted into the wildtype cDNA by site-directed mutagenesis using oligonucleotides containing the mutant sequence. All PCR reactions were carried out using Hot Gyro Polymerase (Solis Biodyne, Tartu, Estonia). Primers used in the study are listed in Supplementary table 1. PCR products were cloned into pTracer-CMV2 as described above.

TRANSFECTION STUDIES

Constructs containing wildtype and different mutant cDNA sequences in pTracer-CMV2 vector were transfected in immortalized patient and control fibroblasts by electroporation as described earlier (9). Rescue of cellular function was tested by measuring the synthesis of methylcobalamin and/or adenosylcobalamin from [^{57}Co]cyanocobalamin, as described previously (11).

RNA EXTRACTION AND REAL-TIME PCR

cDNA was synthesized from total RNA using the TaqMan Reverse Transcription Reagent (Applied Biosystems). Analysis of the abundance of the MMADHC mRNA was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturers recommendations, except that the total reaction volume was decreased to 10 µl. The TaqMan probes (Applied Biosystems) used were Hs00739517_g1 (MMADHC) with Hs00608519_m1 (MRPL19) and Hs99999905_m1 (GAPDH) serving as controls.

IN VITRO TRANSLATION

Wildtype and mutant MMADHC proteins were synthesized *in vitro* using the TNT T7 Quick for PCR DNA kit (Promega, Dübendorf, Switzerland) according to manufacturer's instructions. First, RT-PCR was performed as described above. The specific primers used to produce MMADHC cDNA were modified by inserting the consensus sequence of the T7 promoter to 5' end of the 5' primer and a polyA tail to the 3' end of the 3' primer. The obtained PCR products were used as a template for the TNT reaction. Each reaction contained 20 µl of TNT master mix, 0.5 µl of 1 µM methionine, 2.5 µl of template, 1 µl of biotin-lysyl-tRNA and 25 µl of nuclease-free water. Samples were incubated for 75 min at 30°C. Proteins were separated by SDS-PAGE on a 12 % gel, transferred to polyvinylidene difluoride membranes, and blocked at room temperature for 1 hour with TBS containing 0.1 % Tween 20 (TBS-T) supplemented with 5 % skim milk. Membranes were then incubated with streptavidin-alkaline phosphatase (Promega) for 1 hour. After several washes with TBS-T and distilled water, membranes were incubated with the Western blue substrate (Promega) until the bands became visible. As protein marker we used Kaleidoscope Precision Plus (BioRad, Reinach, Switzerland).

RESULTS

MUTATIONS DETECTED IN cblD PATIENTS

The previous reports of ten patients (9,31) can now be extended by five new patients (Table 1). In these five patients (D010, D012 – D015) we detected five different mutations, 1 of which is novel, c.133dupG, detected in the homozygous state in patient D012. Table 1 summarizes the patients and mutations.

EVALUATION OF FUNCTIONAL CONSEQUENCES OF MUTANT ALLELES

Expression studies in a cblD-MMA/HC cell line confirmed the functional consequences of the novel c.133dupG mutation as well as the c.60insAT, c.683C>G and the c.737A>G mutations described in (31) (see Supplementary Figure 1).

We show that cDNAs containing a mutation associated with cblD-HC phenotype (c.746A>G; c.776T>C) were unable to rescue MeCbl synthesis. MeCbl synthesis could be rescued by constructs containing the c.60insAT or the c.133dupG mutation associated with cblD-MMA phenotype, whereas a construct carrying a nonsense mutation (c.683C>G), associated with cblD-MMA/HC phenotype and the missense mutation c.737A>G associated with cblD-HC phenotype were unable to correct MeCbl synthesis.

Furthermore, we show that 3 of the 4 missense mutation associated with cblD-HC phenotype (c.737A>G, c.746A>G, c.776T>C) rescue AdoCbl synthesis when transfected into cblD-MMA/HC-fibroblasts. The fourth missense mutation (c.545C>A) detected in the heterozygous

state together with c.746A>G in one cblD-HC patient (D002) as well as the nonsense mutation (c.683C>G) associated with the cblD-MMA/HC phenotype did not correct AdoCbl nor MeCbl synthesis indicating that they both represent null mutations.

RESCUE OF AdoCbl SYNTHESIS IN cblD-MMA CELLS

In earlier studies we showed that AdoCbl synthesis was poorly corrected by transfection with the wildtype cDNA, except when using a vector in which a V5-polypeptide was attached to the C-terminus of the wildtype protein (9).

We hypothesized that the low efficiency of rescue of AdoCbl synthesis by the wildtype construct is caused by inefficient mitochondrial targeting. Analysis of the cblD-amino acid sequence by the Mitoprot software (32) predicted a cleavable, N-terminal mitochondrial leader sequence (MLS) of 11 amino acids.

To investigate the efficiency of the endogenous MLS we prepared a wildtype construct (cblD_MLS_ALDH2) in which this putative MLS (Met1-Val12) was replaced by the potent MLS of aldehyde dehydrogenase 2 (ALDH2, NP_000681) which has previously been shown to effectively target natural and synthetic polypeptides to the mitochondria (13). Transfection of this construct into a cblD-MMA/HC (D007) cell line resulted in a dramatic increase of AdoCbl synthesis to high normal or elevated levels (Figure 1), and in a simultaneous decrease of the level of rescue of MeCbl synthesis.

Transfection of cblD-MMA fibroblasts with the cblD wildtype construct resulted in hardly any increase of AdoCbl synthesis, and, in contrast to findings in cblD-MMA/HC cells, transfection with cblD_MLS_ALDH2 failed to normalize AdoCbl synthesis (Figure 2). After Transfection of wt cblD into both cell lines homozygous for c.57_64del (D003 and D010) and into the cell line homozygous for c.133dupG (D012) AdoCbl synthesis increased from 1 % of total cobalamins to only 2.0 – 2.7 % (P=0.025 in D003, P=0.146 in D010 and P=0.073 in D012) whereas transfection of wt cblD into cell lines compound heterozygous for a mutation leading to N-terminal stop and a deleterious mutation in the middle of the gene led to a more prominent increase from 1 % to 3.8 % in D008 (P=0.020) and to 10.5 % in D004 (P=0.004) (Figure 2).

Transfection had no substantial effect on the normal level of MeCbl synthesis in any of the cblD-MMA cell lines (not shown). The exception was cell line D004 in which MeCbl was reduced from 57.9 % to 46.6 % (P=0.044).

Attempts to increase the level of rescue of AdoCbl synthesis with the cblD wildtype cDNA in two cblD-MMA cell lines homozygous for the 57_64del change (D003 and D010) by using another type of vector (pcDNA3) for transient transfection or by using stable transfection in a retroviral system (pBABE) were not successful. When using the retroviral system, again only

a small but statistically significant (P=0.0004) increase of AdoCbl from 1.3 % to 3.4 % was seen (Supplementary Figure 2).

MMADHC mRNA EXPRESSION

Real-time PCR showed considerable differences in the levels of *MMADHC* mRNA in the cblD cell lines (Figure 3). Clearly reduced levels were obtained in D006 a patient with the cblD-MMA/HC phenotype and in D004 with the cblD-MMA phenotype. In all other cell lines the expression varied from 0.6 to 1.4 when compared to that of three control cell lines (normalized to mRNA levels of GAPDH).

There seems to be a negative correlation between the levels of expression of MMADHC mRNA and the level of rescue of AdoCbl synthesis in the different cblD-MMA cell lines described above. Similar results were obtained when the mRNA levels were normalized to the mRNA of the mitochondrial protein MRPL19 (not shown).

EFFECT OF MUTATIONS ASSOCIATED WITH cblD-MMA ON THE LEVEL OF AdoCbl SYNTHESIS

The negative correlation observed between the level of the rescue of AdoCbl synthesis and the level of mRNA among the cblD-MMA cell lines indicates that the endogenous mutant protein in these cells may influence the ability of the wildtype protein to produce AdoCbl. We therefore studied the effect of the four cblD-MMA associated mutations on rescue of AdoCbl synthesis by transfecting a cblD-MMA/HC cell line (D007) simultaneously with cblD_MLS_ALDH2 and constructs carrying cblD-MMA associated mutations (Figure 4).

Cotransfection with empty-vector, the wildtype and a construct containing the nonsense mutation c.683C>G/p.S228X associated with the cblD-MMA/HC phenotype were used as controls.

When compared to co-transfection of cblD_MLS_ALDH2 with empty vector, co-transfection with the c.683C>G construct showed no effect (P=0.267) whereas co-transfection with the wildtype resulted in a significant decrease of AdoCbl synthesis from 18 % of total cobalamins to 12 % (P=0.004). Cotransfection with constructs containing three of the cblD-MMA associated mutations c.57-64del, c.60insAT, and the c.133dupG, reduced the rescue of AdoCbl synthesis to significantly lower levels as compared with the wildtype construct, i.e. to 5.1 % (P=0.0003), 5.9 % (P=0.001) and 8.2 % (P=0.008), respectively. The reduction achieved with the c.160C>T construct (to 10.5 %) did not differ from that obtained with the wildtype construct (P=0.366). When transfecting cblD-HC fibroblasts with these four cblD-MMA associated mutations, a significant drop of MeCbl synthesis with a parallel increase in AdoCbl synthesis was seen with the c.57-64del construct (Supplementary Figure 3).A similar

effect was seen for the c.60insAT and the c.133dupG but not for c.160C>T construct (Supplementary Figure 3).

These findings are in accordance with the variable levels of mRNA and rescue of AdoCbl synthesis in the different cblD-MMA cell lines and indicate that these mutant proteins indeed interfere with the wildtype protein by inhibiting AdoCbl synthesis to various degrees.

REINITIATION OF TRANSLATION

All five patients with the cblD-MMA phenotype have at least one allele carrying a mutation that leads to premature termination of translation before the second methionine located at position 62 (Table 1). The two compound heterozygous patients carry in addition a deletion that leads to frameshift and premature termination at amino acid residue 162 (D008), and an in-frame duplication of the amino acids 103 to 108 (D004), thus mutations that most probably result in an inactive and/or unstable protein. All cblD-MMA patients must at least express a partially active MMADHC protein since MeCbl synthesis is completely normal in these patients. We therefore speculated that there is reinitiation of translation at a downstream AUG codon, e.g. at Met62 (2^{nd} methionine-residue) and/or at Met116 (3^{rd} methionine-residue), which are both are located within a reasonable Kozak consensus sequence (33).

To investigate this we made constructs with selected changes in the cblD wildtype cDNA, transfected them into a cblD-MMA/HC cell line (D007) and investigated the rescue of cobalamin coenzyme synthesis. As shown in Figure 5a transfection with cDNA truncated N-terminally to Met62 or Met116, with cDNA with a stop-codon introduced between Met62 and Met116 (p.S89X), results in the same level of rescue of MeCbl synthesis as transfection with the unchanged wildtype cDNA, whereas no rescue was obtained with cDNA in with codon Gln118 was mutated to a termination codon (p.Q118X). These finding confirm that translation can be initiated not only at the codon for Met1 but also at the codons for Met62 and Met116 but that no initiation of translation occurs downstream of Met116.

We also tested whether reinitiation of translation also occurs in the wildtype: It has been shown that no translational initiation occurs at glutamine residues (24), we therefore tested a cDNA in which both Met62 and Met116 are mutated to glutamine (CAG). Our results show that the full-length protein (containing the endogenous MLS) with no possibility of reinitiation of translation at Met62 and Met116 produced normal levels of MeCbl, suggesting that reinitiation of translation plays a minor role in the wildtype and that the full length protein is dually localized.

As expected transfection with the constructs resulting in deletion of the N-terminal 61 or 115 amino acids were not able to rescue AdoCbl synthesis as they lack a MLS (Figure 5b). However, addition of the MLS of ALDH2 N-terminally to Met62 resulted in rescue of AdoCbl synthesis whereas no rescue was observed when the MLS was attached in front of Met116.

This indicates that the sequence between Met62 and Met116 is essential for AdoCbl synthesis.

CONFIRMATION OF TRANSLATIONAL REINITIATION USING *IN VITRO* PROTEIN SYNTHESIS

Western blot analysis of *in vitro* synthesized proteins (Figure 6) showed that 2 bands are produced when the wildtype cDNA is used as template, one corresponding to the full-length MMADHC protein (32.9 kDa), and the other corresponding to a protein with initiation of translation at Met62 (26.5 kDa).

A shorter protein band with the expected size of 26.5 kDa was obtained from cDNA of a patient with the cblD-MMA phenotype (D003), corresponding to reinitiation of translation at Met62. Shorter protein bands of the expected sizes were also found from cDNA of three patients with the cblD-MMA/HC phenotype, i.e. D007 with predicted protein of 29.8 kDa due to deletion of exon 7 (F204_A232), D005 with predicted protein of 27.8 kDa due to premature termination of translation at R250, and D009 with predicted protein of 25.3 kDa due to premature termination at S228. No detectable protein was obtained when cDNA from patient D006 with a predicted protein of 15.3 kDa due to premature termination of translation at Y140 was used as template.

None of the cblD patients carries mutations between Met62 and Met116, and no protein band corresponding to transcriptional initiation at Met116 (20.5 kDa) was obtained after *in vitro* protein synthesis from cDNA of the patients or controls. However, this short protein could be produced *in vitro* using the construct with cDNA truncated N-terminally till the codon for Met116 as template (not shown). These results indicate that two proteins are produced from the unchanged wildtype cDNA corresponding to translational initiation at Met1 and Met 62.

DISCUSSION

Dual localization of proteins has been shown to be caused by various mechanisms including alternative splicing, initiation codon scan-through, multiple in-frame start codons, incomplete secretion, reverse translocation, spontaneous folding and others (16,18,34-38).

Furthermore proteins that are localized in mitochondria and the cytosol have been shown to have weak mitochondrial leader sequences (MLS) (39). Thus the strength of the mitochondrial leader sequence can shift the balance between mitochondrial and cytosolic localization (12).

We show evidence that the intracellular balance of MMADHC localization can be changed by exchanging the putative endogenous MLS of MMADHC with that of ALDH2 (cblD_MLS_ALDH2). By doing so, the synthesis of mitochondrial AdoCbl increases and

concomitantly cytosolic MeCbl synthesis decreases. Or results suggest that under physiological conditions, there is a delicate balance of intracellular localization of MMADHC.
So far, all patients with the cblD-MMA phenotype carried at least one allele with a nonsense mutation prior to the second methionine located at position 62 of MMADHC. Transcriptional initiation at this position could therefore also explain the dual localization of the MMADHC protein. A full-length protein starting at Met1 could be responsible for AdoCbl synthesis and a shorter protein starting at Met62 for MeCbl synthesis. Another methionine located at position 116 could also act as point of translational reinitiation. To exclude the possibility that MeCbl synthesis is the result of a shorter protein produced, we mutated the second and third ATG to a CAG as it has been shown that this triplet does not serve as an initiation codon (24). When transfecting cblD-MMA/HC cells with this construct we saw no difference in MeCbl synthesis when compared to transfection with the wildtype constuct, suggesting dual localization is really caused by the weak endogenous MLS or by other factors involved in mitochondrial translocation, rather than by transcriptional initiation at multiple AUGs.
To our surprise AdoCbl formation could not be rescued in cblD-MMA patients to the same extent as in cblD-MMA/HC patients, even when using the cblD_MLS_ALDH2 construct. As we observed a negative correlation between the level of endogenous *MMADHC* mRNA and the level of AdoCbl rescue, our results suggest that in these patients the endogenous mRNA and/or MMADHC protein interacts or competes with mRNA or the protein derived from the transfected construct. If MMADHC is part of a complex necessary for the formation of AdoCbl, a shorter MMADHC protein produced in cblD-MMA cells due to reinitiation of translation at Met62 could block formation of functional complexes thereby reducing AdoCbl synthesis. Another possibility is that the short polypeptides that are produced in cblD-MMA patients (from Met1 to the stop codon resulting from the mutation) interacts with the transfected full length protein or with binding partners located in the cytosol or at the mitochondrial membrane.
Double transfection of cblD-MMA/HC cells with the cblD_MLS_ALDH2 and a cblD-MMA construct show the same inhibition. Again co-transfection with the c.160C>T construct had the highest AdoCbl forming activity reflecting the negative correlation between the mRNA stability of this construct with the ability to form AdoCbl.

Using truncated proteins starting at Met62 and Met116 as well as site-directed mutagenesis introducing nonsense mutations at positions 89 (p.S89X) and 118 (p.Q118X), we provide evidence that translational initiation at position 116 is sufficient for MeCbl synthesis. However, up to now, no patient has been reported having an allele with a nonsense mutation between Met62 and Met116. Therefore it remains to be determined if our finding holds true under physiological conditions in a patient carrying such a mutation.

Furthermore by adding the MLS of ALDH2 to the two truncated constructs, our results suggest that the sequence between Met62 and Met116 is important for AdoCbl formation. Physiologically this finding has no implications as a construct starting at Met62 does not have a MLS and is not targeted to mitochondria.

In vitro transcription/translation, supports our hypothesis of reinitiation of translation at Met62, from a wildtype construct two proteins are produced, one starting at Met1, the other starting at Met62. It remains to be determined if this holds also true in control cells when looking at endogenous protein levels. To answer this question, western-blotting will be necessary using a specific anti-MMADHC antibody.

Similarly one would expect a single 26.5 kDa protein being produced from cDNA truncated N-terminally to Met62. Also in a construct in which the codons for Met62 and Met116 are mutated to glutamine one would only expect a single band corresponding to the full length protein.

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No potential conflict of interest relevant to this article was reported.

Table 1: Clinical phenotype, mutations and location of the mutation(s) detected in MMADHC patients.

patient	phenotype	allele 1		allele 2		exon	reference
D003	MMA	c.57_64delCTCTTTAG	p.C19fsX20	c.57_64delCTCTTTAG	p.C19fsX20	3	Pat 3 in DC
D010	MMA	c.57_64delCTCTTTAG	p.C19fsX20	c.57_64delCTCTTTAG	p.C19fsX20	3	this study
D008	MMA	c.60insAT	p.L20fsX21	c.455dupC	p.T152fsX162	3, 5	WG3280 in MO
D012	MMA	c.133dupG	p.A45fsX59	c.133dupG	p.A45fsX59	3	this study
D004	MMA	c.160C>T	p.R54X	c.307_324dup	p.L103_S108dup	4	WG1437 in MO; Pat 4 in DC
D002	HC	c.545C>A	p.T182N	c.746A>G	p.Y249W	6, 8	Pat 2 in DC
D011	HC	c.737A>G	p.D246G	c.737A>G	p.D246G	8	WG3745 in MO
D015	HC	c.737A>G	p.D246G	c.737A>G	p.D246G	8	this study
D013	HC	c.746A>G	p.Y249W	c.746A>G	p.Y249W	8	this study
D014	HC	c.746A>G	p.Y249W	c.746A>G	p.Y249W	8	this study
D001	HC	c.776T>C	p.L259P	c.776T>C	p.L259P	8	Pat. 1 in DC
D006	MMA/HC	c.419dupA	p.Y140X	c.419dupA	p.Y140X	5	Pat 6 in DC
D009	MMA/HC	c.683C>G	p.S228X	c.683C>G	p.S228X	7	WG3583 in MO
D007	MMA/HC	c.696+1_4delGTGA	p.F204_A232del	c.696+1_4delGTGA	p.F204_A232del	intron 7	Pat 7 in DC
D005	MMA/HC	c.748C>T	p.R250X	c.748C>T	p.R250X	8	WG1220 in MO, Pat 5 in DC

Table 1 lists patient number, clinical phenotype, the mutations found on the two alleles as well as the affected exon/intron and the reference in which the patient was first published.

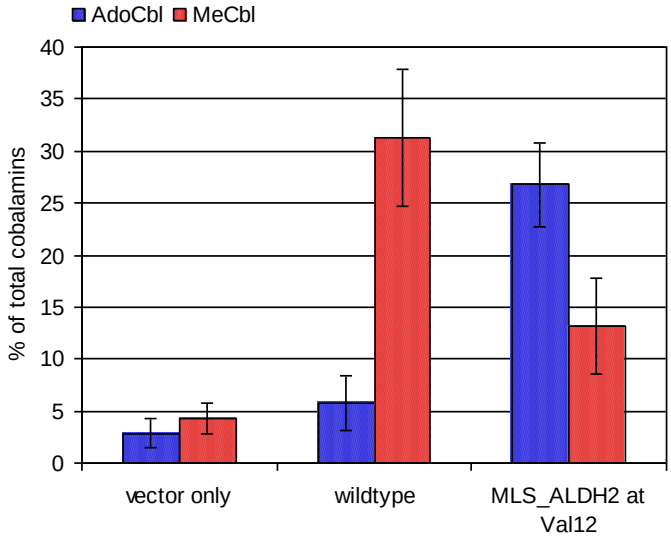


Figure 1: Ado- and MeCbl synthesis after expression of the empty vector, the vector carrying the wildtype *MMADHC* and the vector in which the putative endogenous mitochondrial leader sequence (MLS) of *MMADHC* was replaced with the potent MLS of aldehyde dehydrogenase 2 (ALDH2), in immortalized cblD-MMA/HC fibroblasts (D007).

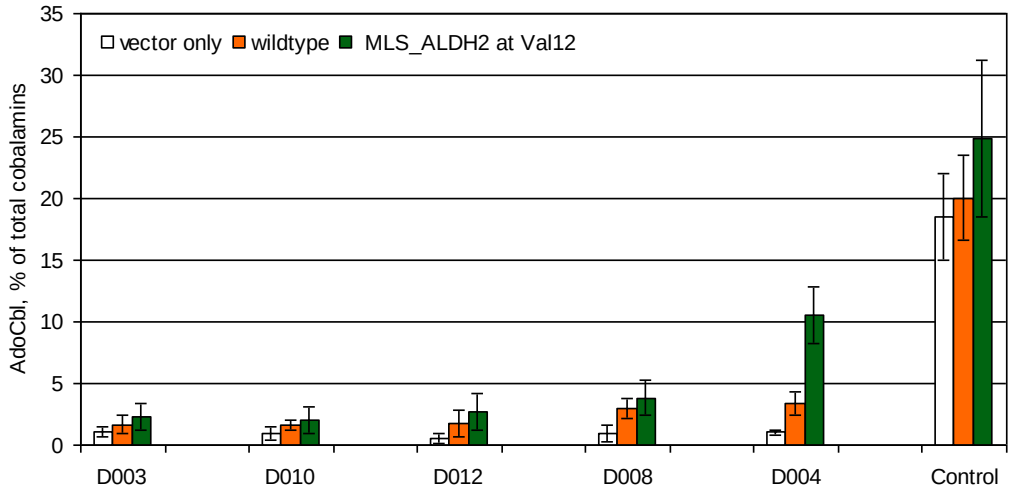


Figure 2: Mitochondrial AdoCbl synthesis after expression of the empty vector, the vector carrying the wildtype *MMADHC* and the vector in which the putative endogenous mitochondrial leader sequence (MLS) of *MMADHC* was replaced with the potent MLS of aldehyde dehydrogenase 2 (ALDH2), in various immortalized cblD-MMA and control fibroblasts.

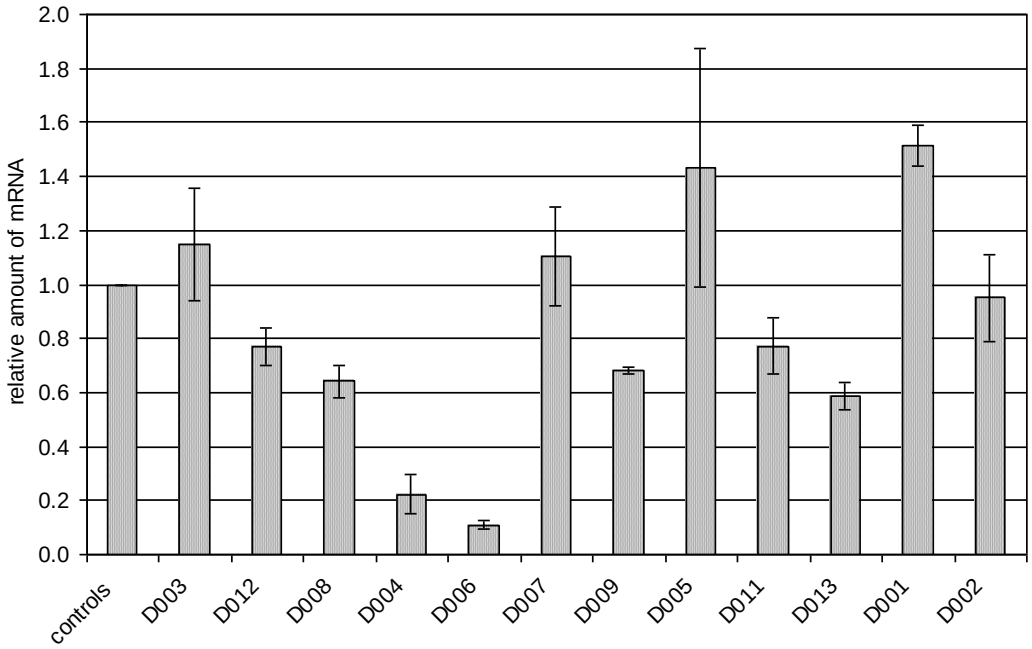


Figure 3: *MMADHC* mRNA levels in various cblD patients. mRNA levels were normalized to levels of *GAPDH* mRNA.

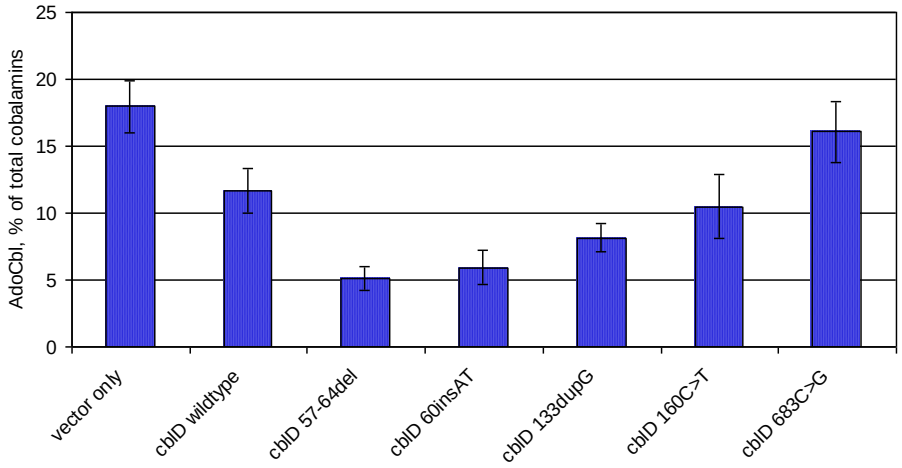


Figure 4: Effect on mitochondrial AdoCbl synthesis after co-expression of the vector in which the putative endogenous mitochondrial leader sequence (MLS) of *MMADHC* was replaced with the potent MLS of aldehyde dehydrogenase 2 (ALDH2), together with the empty and the wildtype vector as well as with vectors carrying mutations associated with cblD-MMA and one associated with cblD-MMA/HC in immortalized cblD-MMA/HC fibroblasts (D007).

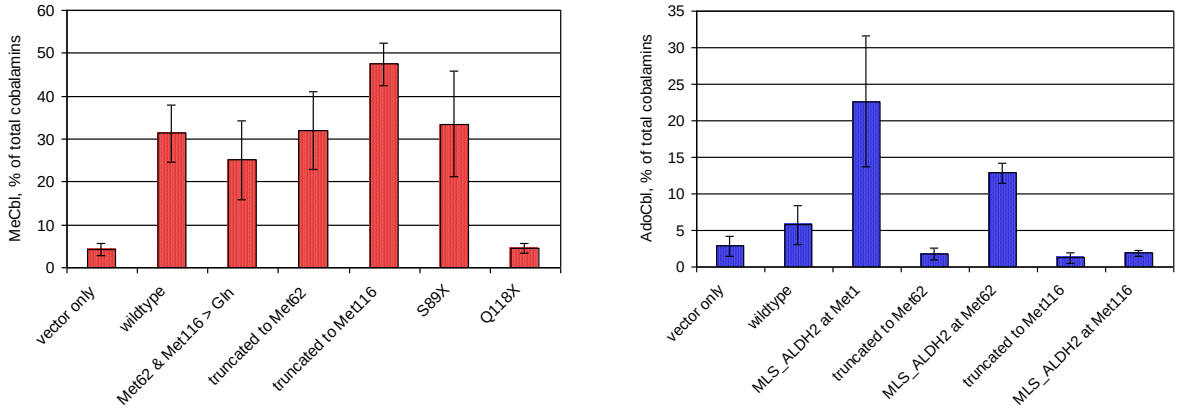
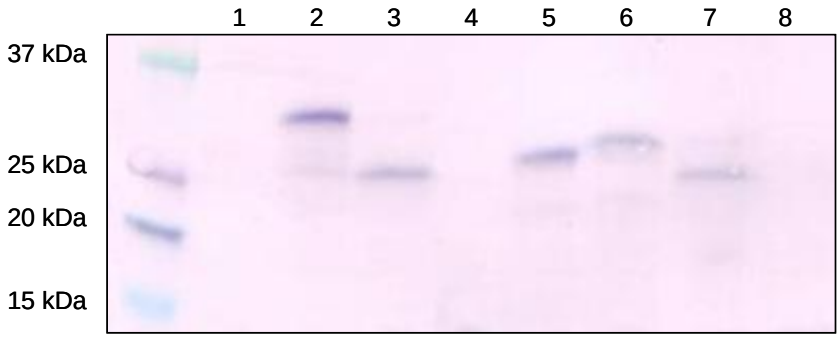
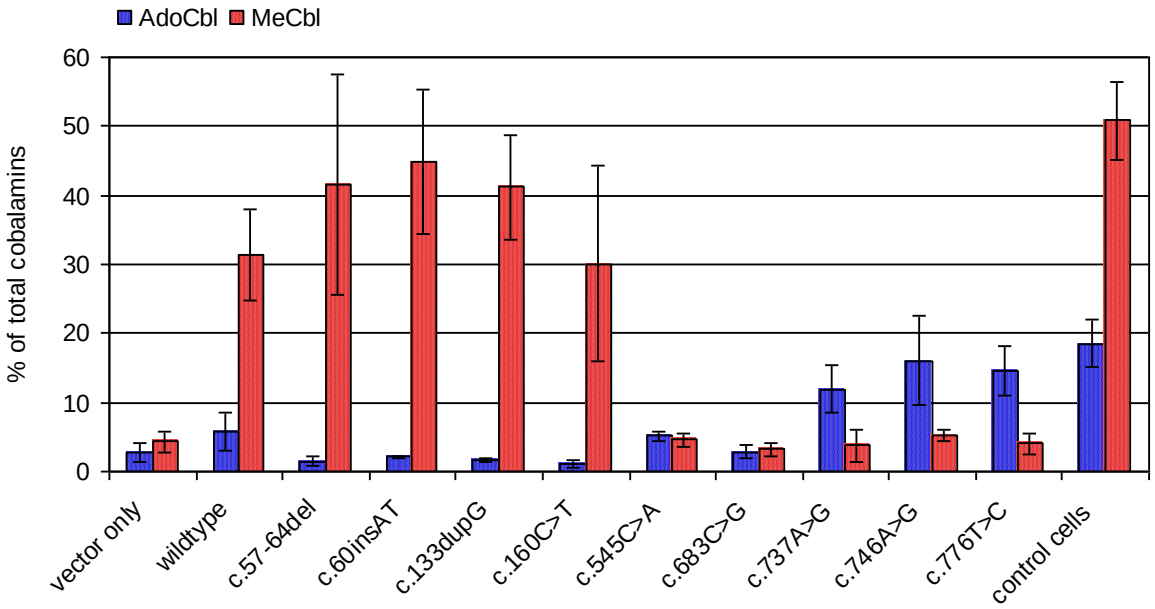


Figure 5: Effect of MeCbl (5a, left) and AdoCbl (5b, right) synthesis after expression of empty vector as well as the vectors carrying the indicated *MMADHC* constructs in immortalized cblD-MMA/HC fibroblasts (D007).

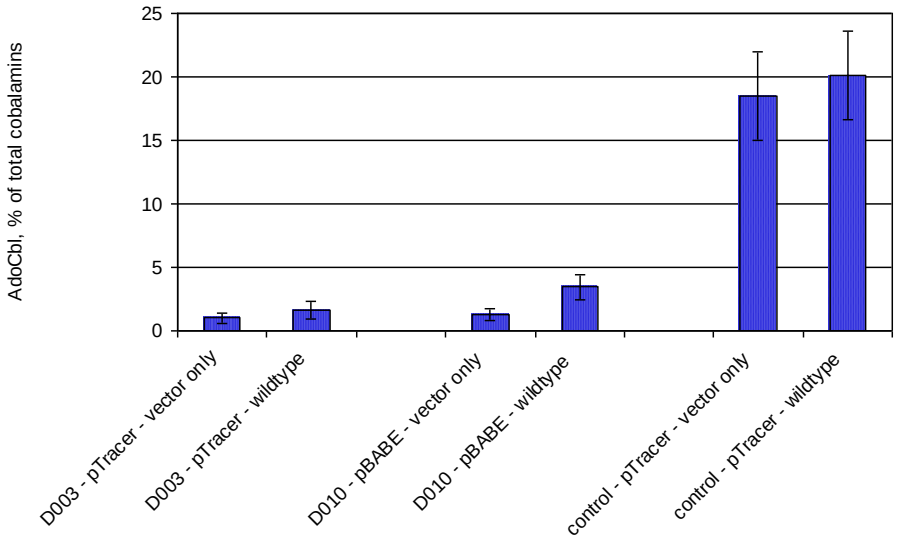


	pTracer	effect on protein	expected size(s)	
1.	vector only		-	
2.	wild-type		32.8 kDa	26.5 kDa
3.	c.57-64del	p.C19fsX20	26.5 kDa	
4.	c.419dupA	p.Y140X	15.1 kDa	8.6 kDa
5.	c.748C>T	p.R250X	27.8 kDa	21.4 kDa
6.	c.696+1_4delGTGA	p.F204_A232del	29.8 kDa	23.3 kDa
7.	c.683C>G	p.S228X	25.3 kDa	18.8 kDa
8.	no DNA		-	

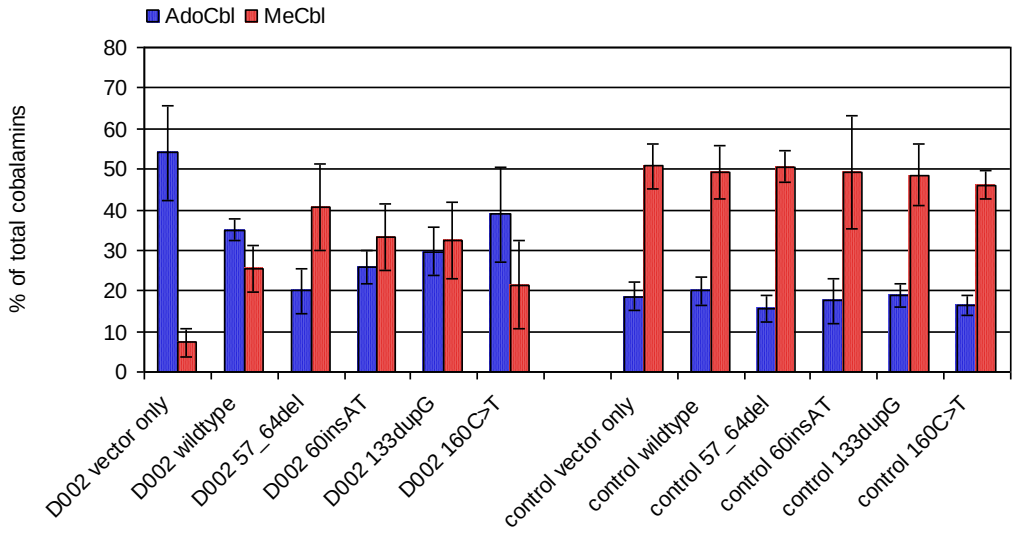
Figure 6: In vitro transcription translation using various pTracer constructs as templates.



Supplementary figure 1: Expression of wildtype and Mutant Alleles of *MMADHC* cblD-MMA/HC fibroblasts (D007).



Supplementary figure 2: Expression of wildtype *MMADHC* in various cblD-MMA fibroblasts (D003, D010) using various expression vectors.



Supplementary figure 3: Expression of wildtype and mutant *MMADHC* alleles associated with the cblD-MMA phenotype into cblD-HC (D002) and control fibroblasts. Effect on synthesis of adenosyl- and methylcobalamin (AdoCbl and MeCbl) synthesis.

Supplementary table 1: Sequences of all primers used in the study.

primer	purpose	sequence (5' - 3')
72F	full-length MMADHC	gttctgggacagctggagac
1260R	full-length MMADHC	caatgtggatgtgttcaacg
581F	sequencing MMADHC constructs	gaaagtgccagagtagagtg
820R	sequencing MMADHC constructs	ccaataaccctcagctcgaa
cblD_NotI_pcDNA_f	V5- and His -tagging (clone into pcDNA3.2/V5)	acatagcggccgccttgttctgggacagc
cblD_AscI_V5_r	V5-tagging (clone into pcDNA3.2/V5)	agataggcgcgccaaatgaatggatatttctgcttatttcc
cblD_AscI_6His_r	6x HIS tagging (clone into pcDNA3.2/V5)	ctatggcgcgccctattaatgatgatgatgatgatgggctaaatttccacttaatttcttcat
ALDH_CblD_out_f	adding MLS of ALDH2	ccgctgcg*atgttgcgcgctgccgcccgcttcgggccccg**cctgggccgccgcctcttgt***
ALDH_CblD_in_Met1_f	adding MLS of ALDH2	gcctgggccgccgcctcttgtcagccgccgccacccaggcc**atg**gccaatgtgctttgta
ALDH_CblD_in_Val12_f	adding MLS of ALDH2	gcctgggccgccgcctcttgtcagccgccgccacccaggcc**gtt**tcctatctcccaggat
ALDH_CblD_in_Met62_f	adding MLS of ALDH2	gcctgggccgccgcctcttgtcagccgccgccacccaggcc**atg**ggacccttggacctc
ALDH_CblD_in_Met116_f	adding MLS of ALDH2	gcctgggccgccgcctcttgtcagccgccgccacccaggcc**atg**gcacaatatgtgaatg
CblD_2ATG_EcoRI_f	truncating MMADHC	acatagaattctgatgaaactatggga
CblD_3ATG_EcoRI_f	truncating MMADHC	acatagaattcaagtgaaagacatga
cblD_2ATG_CAG_f	MMADHC M62Q	ggcctgatgaaactcaggaccctttggacctcaag
cblD_2ATG_CAG_r	MMADHC M62Q	ccaaagggtccctgagtttcatcaggccacactgttc
cblD_3ATG_CAG_f	MMADHC M116Q	gtgaaagacatgagtttgtgcaggcacaatatgtg
cblD_3ATG_CAG_r	MMADHC M116Q	ctgaaattcattcacatattgtgcctgcacaaactcatg
cblD_SDM_S89X_f	MMACHC S89X	ctcaatgggactgcttaacagaagaaaagcctggttc
cblD_SDM_S89X_r	MMACHC S89X	ggcttttcttctgttaagcagtcccattgaggtgacaatc
cblD_SDM_Q118X_f	MMADHC Q118X	gttgtttgtgatggcataatatgtgaatgaatttcagggtaatgatg
cblD_SDM_Q118X_r	MMADHC Q118X	ttcattcacatatttatgccatcacaaactcatgtctttcacttg

Restriction sites are underlined. Mutated triplets are depicted in red. His-tag and MLS ALDH2 are in blue. Overlapping part of outer ALDH2 primer (ALDH_CblD_out_f) is in italics. Bold triplets indicate first triplet of MMADHC.

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OUTLOOK AND CONCLUDING REMARKS

MCC related work

MCC deficiency is increasingly questioned to be a disease as such. Our data provide strong indications that factors other than the genotype at the MCC loci, such as modifying genes and environmental factors, must have a major influence on the phenotype of MCC deficiency (156). In that respect, MCC deficiency is a good example of the now generally accepted idea of complexity in monogenic traits (65).

There are however still numerous MCC-related research projects that could be of interest, they include:

1: The dominant negative mutation found in *MCCA* (p.R385S) leads to a very stable, but non-functional protein (159) To date, our metabolic laboratories in Zurich and Basel have received more than 20 patient cell lines with suspected MCC deficiency, which have MCC activity at or just below the lower normal range associated with a reduced MCC/PCC ratio. Analysis of 2 such patients revealed the *MCCA* p.R385S mutation on one allele. It is possible that also some of the other subjects in this group carry other mutations with a similar effect.

2: Another interesting mutation is the *MCCB* p.224L mutation found in exon 7 of *MCCB*. This exon is spliced out to some extent in all patient and control cells tested so far, raising the question of the importance of exon 7. As exon 7 is in-frame, the deletion of this exon leads to a shorter protein with the same N- and C-terminus as the wild type protein. We have shown that constructs missing exon 7 do not show any rescue of MCC activity when transfected into MCC deficient fibroblasts, nor is any stable protein produced from this construct. It would be interesting to see if this is the case in cells other than fibroblasts as this shorter protein might have some other tissue-specific function.

3: The missense mutation *MCCA* p.M325R was found in exon 10 of a compound heterozygous patient along with another missense mutation *MCCA* p.A289V in exon 8. Analysis of this region of *MCCA* by RT-PCR resulted in 2 transcripts differing in size by 128 bp. Direct sequencing of the 2 products revealed that the transcript of normal length contained the missense mutation in exon 8, while the shorter transcript led to skipping of exon 10 and coded for a normal exon 8. We were able to detect the exon 10 missense mutation p.M325R only by direct sequencing of amplified genomic DNA. We hypothesize that the mutation in exon 10 causes exon skipping because of disruption of an exon splice enhancer sequence (ESE) as it has been shown for the *MCCB* G352R mutation (263).

Finally, research on MCC can serve as an example for other biotinylated enzymes and their disorders. Also the MCC genes can be utilized as model genes for analyzing the effect of various mutations found in our subjects.

Cobalamin related work

Many genes responsible for the different complementation groups of cobalamin metabolism have been described, however, the exact location and function of most of them is still unknown. By describing the dual localization of MMADHC we have shed some light on intracellular cobalamin metabolism. There are still numerous open questions regarding the state of cobalamin after each of the intracellular steps, also the question regarding complex formation or interaction with other proteins of the various proteins involved in intracellular cobalamin metabolism remain unanswered. Interaction partners could possibly be found by using pull-down assays or a yeast-two-hybrid system.

Patients with defects in cobalamin uptake for example should also present with homocystinuria, however publications on these defects do not mention elevated levels of homocysteine. Some case reports of patients with TCII deficiency reported normal levels of plasma homocysteine and amino acid (226). This raises the question on alternative ways of import of cobalamin into cells. This question needs to be addressed in future experiments.

The MMADHC protein has been a nice tool to investigate a protein that serves two functions in two distinct compartments of the cell. Also the balance of localization that could be altered in one or the other way by cleaving and enhancing the MLS has been nice to study. In this respect MMADHC can be a research tool for other proteins with similar distribution patterns and functions. The data presented in chapter 3 of this thesis could not be supported by protein data so far as no antibody was available. A custom made monoclonal antibody is now being tested and will be used to investigate the theory of reinitiation of translation in protein extracts of control and patient fibroblasts as well as in TNT assays. As a recent paper reports that MMADHC could also serve as an adaptor protein for the complexation of MMACHC to acceptor proteins in the cytoplasm and in mitochondria (227), this antibody will be a helpful tool for identifying binding partners of MMADHC.

As a concluding word, I would like to mention that research on rare metabolic disease is important and that a lack of knowledge about these disorders can not only have severe consequences for the subject itself but also on the parents or caretakers as the following (and quite recent) example illustrates:

In 1989 a US woman was sentenced to life in prison after her son had died by suspected ethyleneglycol poisoning, an ingredient of antifreeze. During her time in prison, she gave birth to a second child that was also acutely ill, clinical investigation of the second child revealed high levels of methylmalonic acid. When re-analyzing the sample of the first child, the same finding was made. Methylmalonic acid had been mistaken for ethyleneglycol. In fact, both the first and the second child actually suffered from methylmalonic aciduria (264).

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CURRICULUM VITAE

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Education

01/06	-	12/09	PhD thesis in the Division of Metabolism, University Children's Hospital Zürich
03/05	-	07/05	laboratory work at the Institute of Plant Sciences, ETH Zürich
03/04	-	09/04	Master thesis at the Institute of Medical Microbiology, University of Zürich
10/00	-	04/05	Bachelor and Master, Department of Biology, ETH Zürich; focus on biochemistry, cellbiology, gene-and enzyme-technology and medical microbiology

Publications (prior to my PhD)

- *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance (Antimicrob Agents Chemother. 2006 Apr; 50(4):1183-94)

Courses/Lectures attended during the PhD

- bioethics and biopatents
- business economics for project leaders
- elements of a scientific career strategy
- flow cytometry
- mouse physiology and patophysiology
- scientific writing
- "Young European Biotech Network" careers in life science workshop

Courses taught during the PhD

- circulation
- ergonometry
- membrane transport
- sensory systems
- signal transduction
- various journal-clubs and lab-meetings

Conferences and Meetings (2008-2009)

- poster presentation at the ICIEM in San Diego, USA
- oral presentation at the SGPK in St. Gallen, Switzerland
- poster presentation at the clinical day of research at the University Hospital Zürich
- poster presentation at the SSIEM in Lisbon, Portugal
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- oral presentation at the SSIEM in Hamburg, Germany

Language skills

- languages: German and English: fluent oral and written skills,
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Personal interests

- sports (fitness and endurance training, hiking and swimming)
- photography
- general interest in art, culture, economy, nature and politics